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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of: James P. Elia	)	
	)	Group Art Unit: 1646
Serial No.: 09/836,750	)	
	)	Examiner: Elizabeth C. Kemmerer
Filed: April 17, 2001	)	
	)	
For: METHOD FOR REPAIRING A DAMAGED PORTION OF A HUMAN HEART	)	Client Docket No. 1000-10-CO1

MAIL STOP APPEAL BRIEF-PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPELLANT'S RESPONSE TO
NOTIFICATION OF NON-COMPLIANT APPEAL BRIEF

Dear Sir:

This paper responds to the May 31, 2007 Notification of Non-Complaint Appeal Brief under 37 C.F.R. 41.37, in which the Appeal Brief filed February 23, 2007 was deemed to be defective because, "The brief does not contain copies of the evidence submitted under 37 CFR 1.130, 1.131., or 1.132 or of any other evidence entered by the examiner **and relied upon by appellant in the** appeal, along with a statement setting forth where in the record that evidence was entered by the examiner, as an appendix thereto (37 CFR 41.37(c) (1) (ix))" and "The evidence appendix submitted wit [sic] the appeal brief provided the required statements setting froth where in the record that evidence was entered by the examiner. However, the required copies of the evidence were not submitted."

As required, Appellant hereby submits the required copies, i.e., Items 1-22 cited in the Evidence Appendix.

Appellant believes that the submission of the enclosed copies fully complies with the outstanding Notification of Non-Compliant Appeal Brief.

Respectfully submitted,

Dated: June 6, 2007

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)
)
SERIAL NO.: 09/836,750) EXAMINER: E.C. Kemmerer, Ph.D.
)
FILED: April 17, 2001)
) GROUP ART UNIT: 1646
FOR: METHOD FOR GROWING)
MUSCLE IN A HUMAN HEART)

THIRD SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.

I, Richard Heuser, declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.

2. This Third Supplemental Declaration is submitted in addition to my previous Declaration, dated June 5, 2003; my Supplemental Declaration dated February 4, 2004; and my Second Supplemental Declaration dated July 18, 2004. No changes are made to any of such previous Declarations.

3. My Curriculum Vitae is attached as Exhibit A to my Declaration of June 5, 2003.

4. It is my understanding that the Examiner in charge of the above-identified patent application is also in the Examiner in charge of co-pending patent application Serial No. 09/794,456. In an Advisory Action dated November 26, 2004, for aforesaid Serial No. 09/794456, the Examiner further questioned my qualification to render my opinions in the three previous Declarations mentioned in above Paragraph 2. It is my further

understanding that the Examiner reviewed my U.S. Patent No. 6,190,379 and did not find mention of delivery of any substance to the myocardium nor the word "cell." Also, the Examiner questioned my role in the cell delivery portion of Bioheart's laboratory and clinical trials using skeletal muscle cultured and modified. I provide the following information to respond to the Examiner's newly raised questions.

5. Regarding, U.S. Patent No. 6,190,379, the following is stated in my Second Supplemental Declaration:

> In my U.S. Patent No. 6,190,379 entitled "Hot Tip Catheter," I developed a technique to deliver radiofrequency (PMR). In the full embodiment of the patent, I discuss delivery of protein and/or muscle cells in the myocardium using the inventive technique.

By the above statement, I meant that the device shown in the patent has been used for the delivery of protein and/or muscle cells to the myocardium. At a presentation at the Angiogenesis Meeting in 1999 in Washington, D.C., we described this use of growth factors in a pig model with the development of neo vascularization. Moreover, I have had discussions with Bioheart regarding the use of my U.S. Patent No. 6,190,379 for delivery of cells.

Regarding my work at Bioheart, the following is stated in my Second Supplemental Declaration:

> I have been involved as a member of the scientific advisory board with the world leader in cardiomyocyte regeneration, Bioheart, Miami Lakes, Florida. This company has been involved with laboratory and clinical trials using skeletal muscle cultured and modified. The sample is then delivered into the myocardium via a surgical or catheter approach.

To provide further information regarding the Examiner's questioning my involvement with Bioheart, I am a Scientific Advisory Board Member and in such role advise Bioheart throughout its pre-clinical and clinical work involving the delivery of skeletal muscle

cells into the myocardium. I am also an investigator with Bioheart's Phase 3 clinical trials in the United States. Such trials have not yet commenced.

6. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2/16/05

Richard Heuser, M.D., F.A.C.C., F.A.C.P.

Docket No./~~XXXXXXX~~ 1000-10-C01

Serial No./Reg. No.: 09/836,750

Filed/~~XXXXXXX~~: 04/17/2001

Due Date:

The Patent Office acknowledges, and has stamped hereon, the date of receipt of the items check below:

☒ Transmittal Letter	
☐ Application – Trademark	
☐ Application – Patent Specification	Total Pgs____
☐ Total Claims____ Ind. Claims ____	Total Pgs____
☐ Abstract	Total Pgs____
☐ Drawings: Formal ___ Informal ___	Total Pgs____
☐ Declaration/Oath/Power of Attorney	Total Pgs____
☐ Check No. _____	Fee: $____
☐ Assignment and Cover Sheet	Fee: $____
☐ Request for Non-Publication	
☐ Information Disclosure Statement	
☐ Form PTO-1449 w/Refs____	Total No.
☒ Request for Extension of Time (2 mo)	Fee: $ 225.00
☒ Amendment/Response (Ext. A-O)	
☐ Affidavit/Declaration/Statement	
☐ Brief/Reply Brief/Notice of Appeal	
☐ Fee-Base/Maintenance	Fee: $
☒ Check No. 1120	Fee: $ 700.00
☐ _____	
☐ _____	

Mailed 06/22/2006

EXHIBIT A

Fourth Supplemental Declaration of Dr. Heuser

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)
)
SERIAL NO.: 09/836,750) EXAMINER: Elizabeth C. Kemmerer
)
FILED: April 17, 2001)
) GROUP ART UNIT: 1646
FOR: METHOD FOR GROWING)
MUSCLE IN A HUMAN HEART)

FOURTH SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.

I, Richard Heuser, declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.

2. This Fourth Supplemental Declaration is submitted in addition to my previous Declaration, dated June 5, 2003, my Supplemental Declaration dated February 4, 2004, my Second Supplemental Declaration dated July 18, 2004, and my Third Supplemental Declaration dated February 15, 2005. No changes are made to any of such previous Declarations.

3. My Curriculum Vitae (hereinafter "CV") is attached as Exhibit A to my Declaration of June 5, 2003, and my background is further amplified by materials submitted in my Second and Third Supplemental Declarations.

4. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. Such disclosures are the same as I read and understood in my previous Declaration and Supplemental Declaration. A copy of such disclosures is attached hereto as Fourth Supplemental Declaration Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 37, lines 19-25; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56, line 25; and page 62, lines 1-10. A copy of such additional disclosures is attached hereto as Fourth Supplemental Declaration Exhibit B.

5. The disclosures in Fourth Supplemental Declaration Exhibit A, also contained in my previous Declaration and Supplemental Declaration, relate to using growth factors, including cells, for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new cardiac muscle. Such disclosures are also directed to the growth of new arteries in the heart.

I understand that the additional disclosures in Fourth Supplemental Declaration Exhibit B relate to using cellular growth factors, including bone marrow stem cells, to grow soft tissue, including an artery. Stem cells harvested from bone marrow, peripheral blood and from culture banks are described as being implanted for promoting morphogenesis and growth of all three-germ tissue layers, i.e. mesoderm, ectoderm and endoderm tissues. It would be understood by one skilled in the art that morphogenesis includes the growth of an artery, which comprises mesodermal tissue.

6. I have read and understood the claims set forth in Fourth Supplemental Declaration Exhibit C and have been informed that such claims will be concurrently presented in this application with this Fourth Supplemental Declaration.

7. Based upon above Paragraphs 4-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in the disclosures referenced therein, would be enabled to practice the method set forth in Fourth Supplemental Declaration Exhibit C and to predictably anticipate the results defined therein without need for resorting to undue experimentation. It is my further opinion that one skilled in the art reading such disclosures would understand that all of the well known administration procedures described at page 45 of the patent application, including intravenous, intraluminal, intramuscular, and with an angioplasty balloon, would be applicable for use in growing an artery in a human patient regardless of whether the genetic material was a gene; cell, including stem cells such as bone marrow stem cells; or another type of growth factor.

Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 6/16/00

Richard Heuser, M.D., F.A.C.C., F.A.C.P.

FOURTH SUPPLEMENTAL DECLARATION

EXHIBIT A

DISCLOSURES

EXHIBIT A

DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell

nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles

and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

FOURTH SUPPLEMENTAL DECLARATION

EXHIBIT B

DISCLOSURES

EXHIBIT B

DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 33, LINES 8-10

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 40, LINE 20 – PAGE 43, LINE 3

EXAMPLE 11

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MXS-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

MXS-1 and MXS-2 transcription factors are obtained which will initiate the expression of the MXS-1 and MXS-2 homeobox genes.

The MXS-1 and MXS-2 transcription factors, BMP-2 and BMP-4 bone morphogenic proteins, and MXS-1 and MXS-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 12

Example 11 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 13

Example 11 is repeated except that the MXS-1 and MXS-2 transcription factors are not utilized. The transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

Example 13 is repeated except that the stem cells are starved and the transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 15

WT-1 and PAX genes are obtained from a sample of skin tissue is removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees.

The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factors and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The kidney germ is transplanted in the patient's body near the optic nerve. As the kidney grows, its blood supply will be derived from nearby arteries.

EXAMPLE 17

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 44, LINES 12– 13

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 48, LINES 13– 15

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

PAGE 53, LINE 1 – PAGE 56, LINE 25

EXAMPLE 18

A 36 year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F to produce an genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a

single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting

a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient cultures, physiological nutrient cultures, vectors, promotors, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials inserted ex vivo into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly timed transplantation, organ growth completes itself.

During the ex vivo application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In in vivo or ex vivo embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 19

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace in vivo a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injected intramuscularly. Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736 bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth

factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open heart surgery, endoscopic surgery, direction injection of the needle without incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30^{th} day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both in vitro and in vivo. RAOTS external ultrasound gives a semiquantitative analysis of arterial flow. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 20

A patient, a forty year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five second increments and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 62, LINES 1–10

EXAMPLE 36

Example 18 is repeated except that the patient is a 55 year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

FOURTH SUPPLEMENTAL DECLARATION

EXHIBIT C

CLAIMS

EXHIBIT C

CLAIMS
APPLICATION SERIAL NO. 09/836,750

Claim 236	A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new cardiac muscle and growing a new artery in said heart.
Claim 238	The method of claim 236, further comprising repairing a dead portion of said heart.
Claim 239	The method of claim 236, further comprising repairing a damaged portion of said heart.
Claim 243	The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.
Claim 244	The method of claim 243, wherein said growth factor comprises a cell.
Claim 245	The method of claim 244, wherein said cell is multifactorial and non-specific.
Claim 246	The method of claim 245, wherein said cell comprises a stem cell.
Claim 247	The method of claim 236, wherein said growth factor is placed in said patient by injection.
Claim 248	The method of claim 247, wherein said injection is intravenous.
Claim 249	The method of claim 247, wherein said injection is intraluminal.
Claim 250	The method of claim 247, wherein said injection is intramuscular.

Claim 251 The method of claim 236, wherein said growth factor is placed in said patient by a carrier.

Claim 252 The method of claim 251, wherein said carrier comprises an angioplasty balloon.

Claim 253 The method of claim 236, wherein said growth factor comprises a gene and a cell.

Claim 257 The method of claim 236, wherein said growth factor is locally placed in said body.

Claim 258 The method of claim 238, wherein said growth factor is locally placed in said body.

Claim 259 The method of claim 239, wherein said growth factor is locally placed in said body.

Claim 260 The method of claim 243, wherein said growth factor is locally placed in said body.

Claim 261 The method of claim 236, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 262 The method of claim 238, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 263 The method of claim 239, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 264 A method of growing a new portion of a pre-existing heart comprising locally placing a growth factor comprising a stem cell in a body of a human patient to grow new cardiac muscle in said heart.

Claim 265 The method of claim 264, wherein said stem cell is placed in said patient by injection.

Claim 266 The method of claim 264, wherein said stem cell comprises living stem cells harvested from bone marrow.

Claim 267 The method of claim 266, wherein said stem cell is placed in said patient by injection.

Claim 268 The method of claim 262, wherein said stem cell is placed in said patient by injection.

Claim 269 The method of claim 263, wherein said stem cell is placed in said patient by injection.

Claim 270 The method of claim 258, wherein said growth factor comprises a cell and said cell is placed adjacent to said dead portion of said heart.

Claim 271 The method of claim 259, wherein said growth factor comprises a cell and said cell is placed adjacent to said damaged portion of said heart.

Claim 272 The method of claim 265, wherein said stem cell is injected into said heart.

Claim 273 The method of claim 267, wherein said stem cell is injected into said heart.

Claim 274 The method of claim 238, wherein said growth factor comprises a cell and said cell is placed in said body by intravenous injection.

Claim 275 The method of claim 239, wherein said growth factor comprises a cell and said cell is placed in said body by intravenous injection.

Claim 276 The method of claim 238, wherein said growth factor comprises a cell and said cell is placed in said body by intraluminal injection.

Claim 277 The method of claim 239, wherein said growth factor comprises a cell and said cell is placed in said body by intraluminal injection.

Claim 278 The method of claim 238, wherein said growth factor comprises a cell and said cell is placed in said body by an angioplasty balloon.

Claim 279 The method of claim 239, wherein said growth factor comprises a cell and said cell is placed in said body by an angioplasty balloon.

Claim 280 The method of claim 236 further comprising determining blood flow through said newly grown artery.

Claim 281 The method of claim 238 further comprising determining blood flow through said newly grown artery.

Claim 282 The method of claim 239 further comprising determining blood flow through said newly grown artery.

Claim 283 The method of claim 236 further comprising observing said newly grown artery.

Claim 284 The method of claim 238 further comprising observing said newly grown artery.

Claim 285 The method of claim 239 further comprising observing said newly grown artery.

Claim 286 A method of repairing a dead portion of a pre-existing heart comprising the steps of placing stem cells adjacent said dead portion; forming a new artery in said heart, thereby causing said dead portion of said heart to be repaired.

Claim 287 The method of claim 286, wherein said stem cells are placed by injection.

Claim 288 The method of claim 286, wherein said stem cells are placed by intraluminal administration.

Claim 289 The method of claim 286, wherein said stem cells are placed by an angioplasty balloon.

Claim 290 A method of repairing a damaged portion of a pre-existing heart comprising the steps of placing stem cells adjacent said damaged portion; forming a new artery in said heart, thereby causing said damaged portion of said heart to be repaired.

Claim 291 The method of claim 290, wherein said stem cells are placed by injection.

Claim 292 The method of claim 290, wherein said stem cells are placed by intraluminal administration.

Claim 293 The method of claim 290, wherein said stem cells are placed by an angioplasty balloon.

CERTIFICATE OF MAILING

I hereby certify that the attached DECLARATION OF ANDREW E. LORINCZ, M.D. was delivered to the Assistant Commissioner for Patents by the undersigned from Arrow Intellectual Property Services, 2001, Jefferson Davis Highway, Suite 602, Arlington, Virginia 22202, by hand carrying said DECLARATION to Art Unit 1646, Crystal Plaza 1, Tenth Floor, Attention: Examiner Elizabeth C. Kemmerer this 17th day of June, 2003.

Dated: June 17, 2003

Ann Rutledge

Printed Name: Ann Rutledge

ARROW INTELLECTUAL PROPERTY SERVICE

Docket No./Trademark: 1000-10-C01

Serial No.: 09/836,750

Filed: 04/17/01

Due Date:

The Patent Office acknowledges, and has stamped hereon, the date of receipt of the items check below:

- ☐ Transmittal Letter
- ☐ Application – Trademark
- ☐ Application – Patent Specification Total Pgs____
- ☐ Total Claims____ Ind. Claims ____ Total Pgs____
- ☐ Fee: $____
- ☐ Abstract Total Pgs ____
- ☐ Drawings: Formal __ Informal ___ Total Pgs____
- ☐ Declaration/Oath/Power of Attorney Total Pgs ____
- ☐ Assignment Fee: $____
- ☐ Request for Non-Publication
- ☐ Information Disclosure Statement
- ☐ Form PTO-1449 References ____ Total No ____
- ☐ Request for Extension of Time Fee: $____
- ☐ Amendment/Response
- ☒ Declaration of Andrew Lorincz
- ☐ Brief/Reply Brief/Notice of Appeal
- ☐ Fee-Base/Maintenance Fee: $____
- ☐ Check No. _____ Fee: $____
- ☐ _____
- ☐ _____

COPY

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia

SERIAL NO.: 09/836,750

FILED: April 17, 2001

FOR: METHOD AND APPARATUS FOR INSTALLATION OF DENTAL IMPLANT

EXAMINER: E.C. Kemmerer, Ph.D.

GROUP ART UNIT: 1646

DECLARATION OF ANDREW E. LORINCZ, M.D.

I, Andrew E. Lorincz, declare as follows:

1. I reside at 3628 Belle Meade Way, Mountain Brook, Alabama 35223.

2. My Curriculum Vitae is attached hereto as Exhibit A.

3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. A copy of such disclosures is attached hereto as Exhibit B.

4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue and, more specifically, to a method of using a growth factor for growing muscle in a human heart.

5. I am aware of and have considered the definition of *growth factor* in the specification of the above-referenced patent application at page 20, line 10 through page 21, line 15. Such definition is set forth in Exhibit C along with a definition from the medical dictionary, MEDLINE plus: Merriam-Webster Medical Dictionary. A service of the U.S. NATIONAL LIBRARY OF MEDICINE and the NATIONAL INSTITUTES OF HEALTH. I find that the dictionary definition is consistent with that contained at page 20, line 10 through page 21, line 15 of the above-referenced patent application. I believe that both definitions are appropriate for use in the field of tissue growth and would be understood by one skilled in the medical arts. Accordingly, I am adopting and utilizing the definition contained in the patent application throughout this declaration.

6. I have read and understood the claims set forth in Exhibit D and have been informed that such claims will be presented to the Patent and Trademark Office in the near future.

7. The materials included in attached Exhibit E illustrate that placement of a growth factor in a human patient causes muscle growth in a heart. These materials report work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

8. Based upon above Paragraphs 3-7, it is my opinion that introducing a growth factor into a human patient will predictably cause new muscle growth in the heart of the patient.

9. Based upon above Paragraphs 3-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit D without need for resorting to undue experimentation.

10. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 6-9-03

Andrew E. Lorincz

Andrew E. Lorincz

EXHIBIT A

CURRICULUM VITAE

NAME: Andrew E. Lorincz, M.D.

TITLE: Professor of Pediatrics
University of Alabama at Birmingham

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BIRTH: 5/17/26 Chicago, Illinois

MARITAL STATUS: Married, 12/14/65 - Diane DeNyse Lorincz

EDUCATION:

1948-1952	University of Chicago, School of Medicine, M.D. Degree
1948-1950	University of Chicago, B.S. Degree (Anatomy & Biochemistry)
1946-1948	University of Chicago, Ph.B. Degree

POSTDOCTORAL EDUCATION:

Jan-Mar 1980	Lysosomal Storage Disease Laboratory, Eunice Kennedy Shriver Center, Waltham, MA (Harvard), Visiting Scientist
1955-1956	LaRabida Jackson Park Sanitarium, University of Chicago, Junior Staff Physician Department of Pediatrics, University of Chicago Clinics Bob Roberts Memorial Hospital
1955-1958	Arthritis and Rheumatism Foundation Fellow
1954-1955	Benjamin J Rosenthal Clinical and Research Fellow
1953-1954	Junior Assistant Resident
1952-1953	Intern

ACADEMIC APPOINTMENTS:

1996-present	Professor Emeritus, Department of Pediatrics
1984-1996	School of Public Health, University of Alabama at Birmingham, Professor
1971-1996	University of Alabama at Birmingham, Member of Graduate Faculty
1968-1996	University of Alabama at Birmingham, Professor of Pediatrics
1971-1984	Division of Engineering Biophysics, University of Alabama at Birmingham, Associate Professor
1968-1982	University of Alabama at Birmingham, Associate Professor of Biochemistry
1976- 1980	School of Optometry, University of Alabama at Birmingham, Professor Optometry
1971-1980	School of Nursing, University of Alabama at Birmingham, Clinical Associate Professor
1970-1980	Center for Developmental and Learning Disorders, University of Alabama at Birmingham, (A University Affiliated Facility for Developmental Disability), Director
1970-1976	School of Optometry, University of Alabama at Birmingham, Associate Professor of Pediatric Optometry
1966-1968	Medical Teaching and Research, Unit of the University of Florida at the Sunland Training Center, Gainesville, Florida, Director
1963-1968	Department of Surgery (Orthopaedics), University of Florida College of Medicine, Gainesville, Florida, Research Associate Professor
1962-1968	Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida, Associate Professor
1959-1962	Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida, Assistant Professor
1956-1959	Department of Pediatrics, University of Chicago School of Medicine, Chicago, Illinois, Instructor

PROFESSIONAL LICENSES - PHYSICIAN AND SURGEON:

5/26/69	State of Alabama
8/10/59	State of Florida (inactive)
9/22/54	State of Illinois (inactive)

SPECIALTY CERTIFICATION:

May 1958 American Board of Pediatrics, Diplomate

BOARDS, COMMITTEES AND CONSULTANTSHIPS:

1994-present	Board member of The Mental Retardation and Developmental Disabilities, Health Care Authority of Jefferson County, Inc.
1991-present	Editorial Board for the Annals of Clinical and Laboratory Science, Member
1988-present	Medical Advisory Board of the National MPS Society, Member
1980-1986	Mental Retardation, Consulting Editor
1979-present	National Tay-Sachs and Allied Diseases Association, Scientific Advisory Committee, Member
1978-present	Mayor's Council of Disability Issues
1979-1984	Osteogenesis Imperfecta Foundation, Inc., Board Member Alabama O.I.Chapter
1974-1981	Child Mental Health Services, Inc., Birmingham, Alabama, Board Member
1977-1978	Elizabethtown Committee on Planning and Evaluation, Legislative Committee, State of Pennsylvania
1973-1975	Human Rights Committee for the Partlow State School and Hospital, Tuscaloosa, Alabama, Member - Federal Court Appointed
1971-1974	American Academy of Pediatrics, Committee on Children With Handicaps
1971-1973	American Journal of Mental Deficiency, Consulting Editor
1965-1973	Head Start, Medical Consultant
1967-1972	Journal of Investigative Dermatology, Editorial Consultant
1961-1968	Sunland Hospital, Orlando, Florida, Medical and Research Consultant
1965-1966	State of Florida Interagency Committee on Mental Retardation Planning, Co-Chairman, Mental Retardation Research Committee Alabama Developmental Disabilites Planning Council
1982-1984	Maternal and Child Health, Member of Advisory Committee
1979-1984	Member (Secretary, 1980; Vice Chairman, 1984)
1973-1979	Consultant

American Association of University Affiliated Facilities

1975-1978	American Association of University Affiliated Programs for the Developmentally Disabled, Board Member

American Association on Mental Retardation

1980,84,85	Prevention Committee, Chairman
1980-1982	Member of Council
1978-1980	Medicine Division and Member Executive Committee, Vice President

BOARDS, COMMITTEES AND CONSULTANTSHIPS: (CONT'D)

Association of Retarded Citizens of Jefferson County

1990-present	Board Member
1975-1985	Board Member
1977-1978	Second Vice President
1980	Recipient of Distinguished Service Award

PROFESSIONAL SOCIETIES:

American Academy for Cerebral Palsy and Developmental Medicine, (Fellow)
American Academy on Mental Retardation (President Elect, 1975-76; President, 1976-77)
Emeritus Member
American Academy of Pediatrics (Fellow)
American Association for the Advancement of Science
American Association for Clinical Chemistry, Inc.
American Association on Mental Retardation (Fellow)-Life Member
American Chemical Society
American Federation for Clinical Research
American Medical Association
American Society for Human Genetics
American Society for Investigative Pathology
Association of Clinical Scientists
International Society for Mycoplasmology
Jefferson County Pediatric Society
Society for Complex Carbohydrates
Society for Investigative Dermatology
Society for Pediatric Research
Society for Sigma Xi
Southern Society for Pediatric Research (President, 1964)

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18. Lorincz, A.E.: Screening Measurements of Urinary Acid Mucopolysaccharides for Detection of the Hurler Syndrome. The Clinical Pathology of Infancy, Sunderman, F.W. & Sunderman, F.W., Jr. (Editors), pp. 68-71, 1967.
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EXHIBIT
B

DISCLOSURES

APPLICATION
SERIAL NO. 09/836,750

EXHIBIT B

DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell

nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles

and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

EXHIBIT C

DEFINITIONS

EXHIBIT C

DEFINITIONS

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

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> **Growth factor:** a substance (as a vitamin B_{12} or an interleukin) that promotes growth and especially cellular growth

EXHIBIT D

CLAIMS

EXHIBIT D

CLAIMS

Claim X: **A method for growing a new portion of a pre-existing heart comprising the steps of: placing a growth factor in a body of a human patient and growing new muscle in said heart.**

EXHIBIT E

PUBLICATIONS

EXHIBIT E

PUBLICATION INFORMATION SUMMARY

TITLE	AUTHOR	CITATION	DATE	AUTHOR COUNTRY	ROUTE OF ADMINISTRATION	GROWTH FACTOR ADMINISTERED	RESULT
Left Ventricular Electromechanical Mapping to Assess Efficacy of phVEGF165 Gene Transfer for Therapeutic Angiogenesis in Chronic Myocardial Ischemia	Vale	Circulation. 2000; 102:965-974	08/29/00	U.S.	Small incision (minithoracotomy) with syringe injection	VEGF (Gene form)	Repair of damaged portion of heart – Also pertains to new muscle growth
Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans	Strauer	Circulation. 2002; 106:1913-1918	10/08/02	Germany	Balloon catheter with injection	Bone Marrow Cells	Repair of dead portion of heart – also pertains to new muscle growth

TITLE	AUTHOR	CITATION	DATE	AUTHOR COUNTRY	ROUTE OF ADMINISTRATION	GROWTH FACTOR ADMINISTERED	RESULT
Viability and differentiation of autologous skeletal myoblast grafts in ischemic cardiomyopathy	Hagege	Lancet 2003 Feb 8; 361 (9356):491-492	2003	France	Injection	Skeletal Muscle Cells	Repair of dead portion of heart; Histological Proof (muscle)
Autologous Cell Transplant Helpful in Ischemic Heart or Legs	Barclay	Medscape Medical News 2000 – Abstract from American Heart Association's 75th Scientific Sessions on 11/18/02, Chicago	11/18/02	U.S.	Surgery with syringe injection	Bone Marrow Cells	Repair of damaged portion of heart – also pertains to new muscle growth
Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation	Pagani	J Am Coll Cardiol 2003 Mar 5; 41(5): 879-888	2003	U.S.	Surgery with syringe injection	Skeletal Muscle Cells	Repair of dead portion of heart; Histological Proof (muscle and blood vessels)

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

Bodo E. Strauer, MD; Michael Brehm, MD; Tobias Zeus, MD; Matthias Köstering, MD; Anna Hernandez, PhD; Rüdiger V. Sorg, PhD; Gesine Kögler, PhD; Peter Wernet, MD

Background—Experimental data suggest that bone marrow–derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30±13 to 12±7%, $P=0.005$) and was also significantly smaller compared with the standard therapy group ($P=0.04$). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, $P=0.028$). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (*Circulation*. 2002;106:1913-1918.)

Key Words: myocardial infarction ■ cell transplantation, intracoronary ■ angiogenesis ■ bone marrow ■ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.[1,2] Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.[3]

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.[4-7] Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow–derived cells. Bone marrow contains multipotent adult stem cells that show a high capacity for differentiation.[8-10] Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.[11-14] However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.[15] Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.[16] However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

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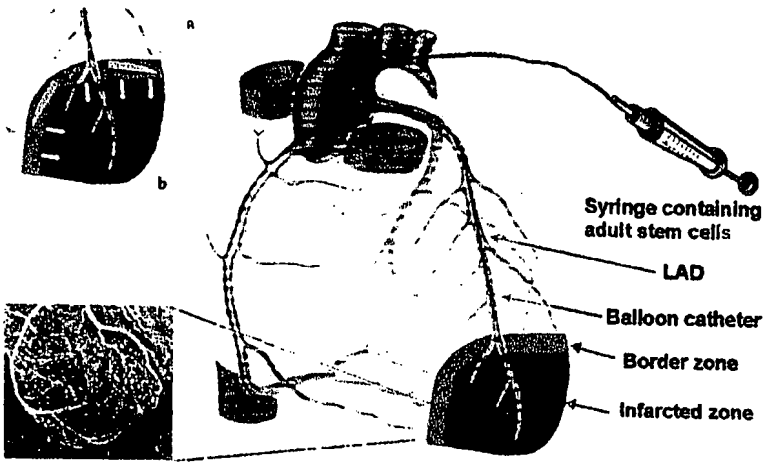

Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. a, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. b, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. c, A supply of blood flow exists within the infarcted zone.[35] The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.[17] To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery (n=4), left circumflex coronary artery (n=3), or right coronary artery (n=13). Mean duration of infarct pain was 12±10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty (n=20) and subsequent stent implantation (n=19). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β-blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (±2) days after acute coronary angiography, bone marrow (~40 mL) was aspirated under local anesthesia from ilium of cell therapy patients (n=10). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H_2O. For overnight cultivation, 1×10^6 BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was 93±3%. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10^7; this consisted of 0.65±0.4% AC133-positive cells and 2.1±0.28% CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality ex vivo control, 1×10^5 cells grown in H5100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.[18]

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.[19] All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radionuclide ventriculography, catheterization of the right heart, and

TABLE 1. Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	P
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2	...	...
Mononuclear bone marrow cells, n ($\times 10^7$)	2.8±2.2	...	...

Values are mean±SD or number of patients.
NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection ^{201}thallium scintigraphy. The contractility index P_{sys}/ESV was calculated by dividing LV systolic pressure (P_{sys}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypokinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, P=0.005). It was also significantly smaller compared with the standard therapy group after 3 months (P=0.04). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, P=0.028) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, P=NS). No significant difference was observed between the two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm², P=0.016, assessed by ^{201}thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

(1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m², P=0.010) and ejection fraction (from 51±14 to 53±13%, P=NS).

(2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, P=NS) and end-systolic volume (from 82±26 to 67±21 mL, P=0.011). Radionuclide ventriculography was used to acquire the data.

(3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, P=NS, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, P=0.005).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	*P*
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dyskinetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dyskinetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow–derived cells in experimental infarction.[11–14,18,20–23] Moreover, transendothelial migration from the coronary capillaries and incorporation of cells into heart muscle has been observed experimentally.[3,12,24–26] Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.[15,16] Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.[27] Adult, mononuclear BMCs contain such stem and progenitor cells (≤1%), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

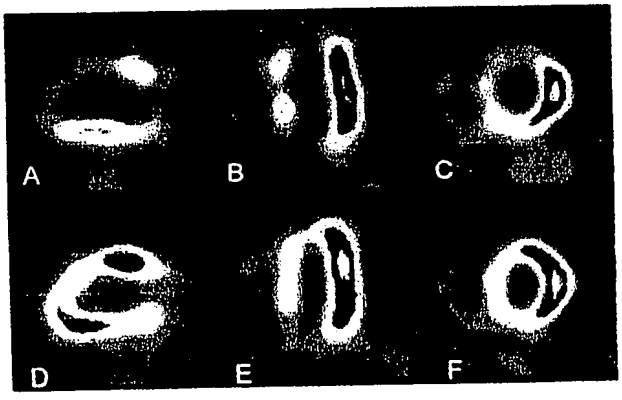

Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by ^{201}thallium scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	*P*
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume index, mL/m^2	49±7	56±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P_{syst}/ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
^{201}Thallium scintigraphy, cm^2	174±99	128±71	0.016

NS indicates not significant.

and smooth muscle cells[8-13]; (3) BMCs give rise to mesodermal progenitor cells that differentiate to endothelial cells[28]; and (4) endothelial progenitors can transdifferentiate into beating cardiomyocytes.[29] Thus, several different fractions of mononuclear BMCs may contribute to the regeneration of necrotic myocardium and vessels. In order to utilize this large and perhaps heterogeneous regenerative potential, we decided to use all mononuclear cells from the bone marrow aspirate as a whole, rather than a subpopulation. No further expansion was performed because experimental data have revealed a dramatic decline in the homing capacity of in vitro amplified hematopoietic stem or progenitor cells.[30]

The second question was how to deliver the cells most efficiently. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection: assuming a normal coronary blood flow of 80 mL/min per 100 g of LV weight, a quantity of 160 mL per left ventricle (assuming a regular LV mass of ≈200 g) will flow per minute.[31,32] This corresponds to only about 3% of cardiac output (assuming a cardiac output of 5000 mL/min).[31] Therefore, intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Thus, supplying the entire complement of cells by intracoronary administration obviously seems to be advantageous for the tissue repair of infarcted heart muscle and may also be superior to intraventricular injection,[33] because all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage. Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched with the maximum available amount of cells at all times.

As stem cells differentiate into more mature types of progenitor cells, it is thought that a special microenvironment in so-called niches regulates cell activity by providing specific combinations of cytokines and by establishing direct cellular contact. For successful long-term engraftment, at least some stem cells have to reach their niches, a process referred to as homing. Mouse experiments have shown that significant numbers of BMCs appear in liver, spleen, and bone marrow after intravenous injection.[34] To offer the BMCs the best chance of finding their niche within the myocardium, a selective intracoronary delivery route was chosen. Presumably, therefore, fewer cells were lost by extraction toward organs of secondary interest by this first pass–like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area, and the balloon was kept inflated for 2 to 3 minutes; the cells were not washed away immediately under these conditions.

The time point for delivery was chosen as 7 to 8 days after infarction onset for the following reasons:

(1) In dogs, infarcted territory becomes rich in capillaries and contains enlarged, pericyte-poor "mother vessels" and endothelial bridges 7 days after myocardial ischemia and reperfusion. Twenty-eight days later, a significant muscular vessel wall has already formed.[35] Thus, with such timing, cells may be able to reach the worst damaged parts and at the same time salvage tissue. Transendothelial cell migration may also be enhanced because an adequate muscular coat is not yet formed.

(2) Until now, only one animal study has attempted to determine the optimum time for cardiomyocyte transplantation to maximize myocardial function after LV injury. Adult rat hearts were cryoinjured and fetal rat cardiomyocytes were transplanted immediately, 2 weeks later, and 4 weeks later. The authors discussed the inflammatory process, which is strongest in the first days after infarction, as being responsible for the negative results after immediate cell transplantation, and they assumed that the best results seen after 2 weeks may have been due to transplantation before scar expansion.[36] Until now, however, no systematic experiments have been performed with BMCs to correlate the results of transplantation with the length of such a time delay.

(3) Another important variable is the inflammatory response in MI, which seems to be a superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. In the first 48 hours, debridement and formation of a fibrin-based provisional matrix predominates before a healing phase ensues.[37-40] Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the decline of adhesion molecules (intercellular adhesion molecules, vascular cell adhesion molecules) does not take place before days 3 to 4 after MI. We assumed that transplantation of mononuclear BMCs within the "hot" phase of post-MI inflammation might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels.

Taking all of this into account, we can conclude that cell transplantation within the first 5 days after acute infarction is not possible for logistical reasons and is not advisable because of the inflammatory process. On the other hand, transplantation 2 weeks after infarction scar formation seems to reduce the benefit of cell transplantation. Although the ideal time point for transplantation remains to be defined, it is most likely between days 7 and 14 after the onset of MI, as in the present study.

This trial was designed as a phase I safety and feasibility trial, meaning that no control group is necessarily required. However, to validate the results, we correlated them with those obtained from 10 patients who refused to get additional cell therapy and thus received standard therapy alone. We are aware of the fact that such a comparison does not reach the power of a randomly allocated, blinded control group. However, the significant improvement with regard to infarct region, hemodynamics (stroke volume index), cardiac geometry (LV end-systolic volume), and contractility (P_{sys}/ESV and infarction wall movement velocity) did confirm a positive effect of the additional cell therapy because the changes observed in the standard therapy group failed to reach significance.

Another important factor for interpreting the results is time interval between onset of symptoms and revascularization of the infarct-related artery by angioplasty; this represents a crucial determinant of LV recovery. For patients with acute MI, it has

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.[41] None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12±10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.[41] In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angioneogenesis and cardiomyoneogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

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Viability and differentiation of autologous skeletal myoblast grafts in ischaemic cardiomyopathy.

Hagege AA, Carrion C, Menasche P, Vilquin JT, Duboc D, Marolleau JP, Desnos M, Bruneval P.

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Autologous skeletal myoblast transplantation might improve postinfarction ventricular function, but graft viability and differentiation (ie, proof of concept) has not been shown. A 72-year-old man had autologous cultured myoblasts from his vastus lateralis injected to an area of transmural inferior myocardial infarction in non-reperfused scar tissue. He showed improvement in symptoms and left-ventricular ejection fraction. When he died 17.5 months after the procedure, the grafted post-infarction scar showed well developed skeletal myotubes with a preserved contractile apparatus. 65% of myotubes expressed the slow myosin isoform and 33% coexpressed the slow and fast isoforms (vs 44% and 0.6%, respectively, in skeletal muscle). Myoblast grafts can survive and show a switch to slow-twitch fibres, which might allow sustained improvement in cardiac function.

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myocardial tissue. These results establish the feasibility of myoblast transplants for myocardial repair in humans.

Publication Types:
- Clinical Trial
- Clinical Trial, Phase I

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Medscape
Medical News

Autologous Cell Transplant Helpful in Ischemic **Heart** or Legs

Laurie Barclay, MD

Nov. 18, 2002 — Autologous cell transplantion may benefit ischemic hearts and legs, according to three presentations on Nov. 18 at the American **Heart** Association's 75th Scientific Sessions held in Chicago, Illinois. Two studies focused on injecting autologous bone marrow cells or autologous skeletal myoblasts into the scarred area of an infarcted **heart**. In another study, injecting autologous bone marrow into ischemic limbs led to new vessel growth, reducing the need for amputation.

"Bone marrow not only can differentiate into **heart** cells, but also smooth muscle cells, connective **tissue** cells and other types of cells to reconstitute the entire structure of a **tissue**," presenter Manuel Galinanes, MD, from the University of Leicester in the U.K., says in a news release. "The benefit [of transplanting bone marrow into scar **tissue** of the **heart**] could be seen only six weeks **after** injection."

In 14 patients with low ejection fraction post-myocardial infarction (MI), autologous bone marrow from the sternum was injected into scarred myocardium during nonemergency coronary artery bypass surgery. **Heart** wall motion measured with echocardiography improved within weeks of treatment, and improvements persisted for at least 10 months **after** treatment.

The regional wall motion score decreased significantly, reflecting less movement abnormality, from a mean score of 2.41 at baseline to 2.16 six weeks **after** treatment and 2.09 ten months **after** treatment. The global wall motion score also decreased significantly from 1.96 before surgery to 1.64 at six weeks, and stabilized at 1.65 **after** 10 months.

Although it is still unproven that bone marrow creates a new cellular infrastructure in **heart** scar **tissue**, "that is the only possible explanation," Galinanes says. "The ability to confirm the presence of scar **tissue** with dobutamine stress echo before surgery, and then confirm it again during surgery, told us that the affected area was dysfunctional and the abnormality was irreversible. We wanted to make sure that we were injecting the marrow into **dead tissue** to help ensure that the injection would not pose any serious risk to the patient."

If additional studies confirm safety and efficacy, Galinanes says that this treatment would be a welcome addition to the post-MI arsenal, which also includes gene therapy, growth factor therapy, and laser treatments.

In a multicenter trial supervised by the U.S. Food and Drug Administration, investigators safely transplanted 16 patients with autologous skeletal myoblasts injected into hearts severely damaged by MI or **heart** failure. Baseline left-ventricular ejection fraction was less than 30%. Eleven patients were undergoing coronary artery bypass surgery and five were having implantation of a left ventricular assist device. Myoblasts extracted from thigh muscle were grown in large quantities in vitro using a controlled cell expansion manufacturing process, and were injected in doses ranging from 10 million to 300 million cells.

"We have been able to **regenerate dead heart** muscle, or scar **tissue**, in the area of **heart** attack without increasing risk of death. Our findings will allow us to move forward with testing if the procedure can improve the contractility of the **heart**," says lead author Nabil Dib, MD, from the Arizona **Heart** Institute in Phoenix. "We found that the transplanted myoblasts survived and thrived in patients. Areas damaged by **heart** attack and cardiovascular disease showed evidence of repair and viability."

Twelve weeks **after** transplant, mean ejection fraction rates improved from 22.7% to 35.8%, or a 58% increase. Echocardiogram, magnetic resonance imaging, and positron emission tomography showed evidence of regeneration in the area of the graft. There were no significant adverse events related to the cell transplant procedure at nine-month follow-up.

The third study showed that bone marrow cells implanted into ischemic legs in patients with peripheral arterial disease (PAD) formed new blood vessels, increased blood flow, and prevented amputation.

"This is the first multicenter and double-blind clinical study to prove the clinical efficacy of growing new blood vessels (angiogenesis) using bone marrow cell transplantation," says lead author Hiroya Masaki, MD, PhD, from Kansai Medical University in Osaka, Japan.

In this randomized trial, 45 patients with PAD received injections of autologous bone marrow mononuclear cells into the calf muscles. Compared with controls who received saline injections, patients who received bone marrow mononuclear cell transplants had a "striking" increase in new capillary formation and in newly visible collateral vessels.

Of 45 treated patients, 31 had an increase in ankle-brachial pressure index in the treated limbs, and 39 had decreased rest pain with improved treadmill endurance. Ischemic ulcers or gangrene healed in 21 of 28 treated limbs.

CD34-cells, which can develop into endothelial progenitor cells, expressed angiogenic growth factors including basic fibroblast growth factor, vascular endothelial growth factor, and angiopoietin-1. Although more research is needed to determine long-term efficacy and safety, "this new angiogenesis therapy using bone marrow cell transplantation may help many patients suffering with ischemic limbs," Masaki says.

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Reviewed by Gary D. Vogin, MD

1: J Am Coll Cardiol 2003 Mar 5;41(5):879-88

ELSEVIER SCIENCE FULL-TEXT ARTICLE

Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation.

Pagani FD, DerSimonian H, Zawadzka A, Wetzel K, Edge AS, Jacoby DB, Dinsmore JH, Wright S, Aretz TH, Eisen HJ, Aaronson KD.

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OBJECTIVES: We report histological analysis of hearts from patients with end-stage heart disease who were transplanted with autologous skeletal myoblasts concurrent with left ventricular assist device (LVAD) implantation. BACKGROUND: Autologous skeletal myoblast transplantation is under investigation as a means to repair infarcted myocardium. To date, there is only indirect evidence to suggest survival of skeletal muscle in humans. METHODS: Five patients (all male; median age 60 years) with ischemic cardiomyopathy, refractory heart failure, and listed for heart transplantation underwent muscle biopsy from the quadriceps muscle. The muscle specimen was shipped to a cell isolation facility where myoblasts were isolated and grown. Patients received a transplant of 300 million cells concomitant with LVAD implantation. Four patients underwent LVAD explant after 68, 91, 141, and 191 days of LVAD support (three transplant, one LVAD death), respectively. One patient remains alive on LVAD support awaiting heart transplantation. RESULTS: Skeletal muscle cell survival and differentiation into mature myofibers were directly demonstrated in scarred myocardium from three of the four explanted hearts using an antibody against skeletal muscle-specific myosin heavy chain. An increase in small vessel formation was observed in one of three patients at the site of surviving myotubes, but not in adjacent tissue devoid of engrafted cells. CONCLUSIONS: These findings represent demonstration of autologous myoblast cell survival in human heart. The implanted skeletal myoblasts formed viable grafts in heavily scarred human

CERTIFICATE OF MAILING

I hereby certify that the attached AMENDMENT (with Exhibits I-IX and two IDS documents); Supplemental Declaration of Richard Heuser; and Supplemental Declaration of Andrew E. Lorincz were delivered to the Commissioner for Patents by the undersigned from Arrow Intellectual Property Services, 2001, Jefferson Davis Highway, Suite 602, Arlington, Virginia 22202, by hand carrying said AMENDMENT to ~~Art Unit 1646~~ MAIL Room, Attention: Examiner Elizabeth C. Kemmerer this 17th day of February, 2004.

Dated: 2/17/04

Ann Rutledge

Printed Name: Ann Rutledge

ARROW INTELLECTUAL PROPERTY SERVICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia	)	
	)	
SERIAL NO.: 09/836,750	)	EXAMINER: E.C. Kemmerer, Ph.D.
	)	
FILED: April 17, 2001	)	
	)	GROUP ART UNIT: 1646
FOR: METHOD FOR GROWING MUSCLE IN A HUMAN HEART	)	

SUPPLEMENTAL DECLARATION OF ANDREW E. LORINCZ, M.D.

I, Andrew E. Lorincz, declare as follows:

1. I reside at 13820 NW County Rd 235, Apt 8, Alachua, FL 32616-2098.

2. This Supplemental Declaration is submitted in addition to my previously submitted Declaration in this application, dated June 9, 2003, and makes no changes to such previous Declaration.

3. My Curriculum Vitae is attached as Exhibit A to my previous Declaration.

4. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. Such disclosures are the same as read and understood by me in my previous Declaration. A copy of such disclosures is attached hereto as Supplemental Exhibit A.

5. I note that the disclosures referenced in above Paragraph 4 relate to using a growth factor for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new muscle in the heart.

6. I have read and understood the claims set forth in Supplemental Exhibit B and have been informed that such claims will be presented to the Patent and Trademark Office in the near future.

7. Based upon above Paragraphs 4-6 and Paragraph 7 of my previous Declaration, it is my opinion that introducing a growth factor into a human patient will predictably cause new muscle growth in the heart of the patient.

8. Based upon above Paragraphs 4-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Supplemental Exhibit B without need for resorting to undue experimentation. I have been informed that the Examiner has questioned the fact that dosages are not recited in the specification of the above-identified application in connection with the administration of cell growth factors to a human patient with use of intravenous or intraluminal techniques. Such techniques are the subject of claims 248-249 in above-mentioned Supplemental Exhibit B. In my opinion, dosages of cellular growth factors to achieve the above-mentioned heart muscle growth are a matter of routine medical practice, requiring only a reasonable degree of experimentation, depending upon such factors as extent of prior heart condition, size of patient, age of patient, health of patient, etc. Consequently, it is my opinion that the disclosure mentioned in Supplemental Exhibit A would enable a person skilled in the medical arts to practice the invention of claims 248-249 and predictably anticipate the results defined therein without need for resorting to undue experimentation.

9. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2-3-04

Andrew E. Lorincz

SUPPLEMENTAL EXHIBIT A

DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell

nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles

and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

SUPPLEMENTAL EXHIBIT B

CLAIMS
APPLICATION SERIAL NO. 09/836,750

236. A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new muscle and growing a new artery in said heart.

238. The method of claim 236, further comprising repairing a dead portion of said heart.

239. The method of claim 236, further comprising repairing a damaged portion of said heart.

240. The method of claim 236, wherein said growth factor comprises genetic material selected from the group consisting of a portion of a gene, a gene, a gene product, and an extracellular matrix.

241. The method of claim 240, wherein said genetic material comprises a gene.

242. The method of claim 241, wherein said gene comprises VEGF.

243. The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.

244. The method of claim 243, wherein said growth factor comprises a cell

245. The method of claim 244, wherein said cell is multifactorial and non-specific.

246. The method of claim 245, wherein said cell comprises a stem cell.

247. The method of claim 236, wherein said growth factor is placed in said patient by injection.

248. The method of claim 247, wherein said injection is intravenous.

249. The method of claim 247, wherein said injection is intraluminal.

250. The method of claim 247, wherein said injection is intramuscular.

251. The method of claim 236, wherein said growth factor is placed in said patient by a carrier.

252. The method of claim 251, wherein said carrier comprises an angioplasty balloon.

253. The method of claim 236, wherein said growth factor comprises a gene and a cell.

Docket No. 1000-10-C01

Serial No. 09/836,750

Filed 04/17/01

Due Date: - - -

The Patent Office acknowledges, and has stamped hereon, the date of receipt of the items check below:

- [X] Transmittal Letter
- [ ] Application – Trademark
- [ ] Application – Patent Specification — Total Pgs____
- [ ] Total Claims____ Ind. Claims____ — Pgs____
- [ ] Abstract — Total Pgs____
- [ ] Drawings: Formal ___ Informal ___ — Total Pgs____
- [ ] Declaration/Oath/Power of Attorney — Total Pgs____
- [ ] Check No. _____ — Fee: $_____
- [ ] Assignment and Cover Sheet — Fee: $_____
- [ ] Request for Non-Publication
- [ ] Information Disclosure Statement
- [ ] Form PTO-1449 w/Refs_____ — Total No. ____
- [ ] Request for Extension of Time — Fee: $_____
- [ ] Amendment/Response
- [X] ~~Affidavit~~/Declaration ~~of~~ 2nd Supplemental of Drs. Heuser and Lorincz
- [ ] Brief/Reply Brief/Notice of Appeal
- [ ] Fee-Base/Maintenance — Fee: $_____
- [ ] Check No. _____
- [X] w/Exhibits A - B
- [ ] _____

07/26/04

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia)
)
Serial No.: 09/836,750)
)
Filed: April 17, 2001)
)
For: METHOD FOR GROWING)
MUSCLE IN A HUMAN HEART)

Group Art Unit: 1646

Examiner: Elizabeth C. Kemmerer, Ph.D.

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail, in an envelope addressed to Mail Stop Non-Fee Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on July 26, 2004

Gerald K. White 7/26/04
Signature Date

LETTER

Mail Stop Non-Fee Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This Letter is submitted in an attempt to advance the prosecution of the instant patent application.

In related patent application Serial No. 09/794,456, that is currently being examined by the Examiner in charge of this application, Second Supplemental Declarations of Drs. Richard Heuser and Andrew E. Lorincz were prepared and will be filed in the near future. Accordingly, Applicant wishes to file Second Supplemental Declarations in this application as well.

Should the Examiner have any questions or require additional information or discussion to place the application in condition for allowance, a phone call to the undersigned attorney would be appreciated.

Respectfully submitted,

Date: July 26, 2004

Gerald K. White
Reg. No. 26,611
Attorney for Applicant

GERALD K. WHTIE & ASSOCIATES, P.C.
205 W. Randolph Street, Suite 835
Chicago, IL 60606
Phone: (312) 920-0588
Fax: (312) 920-0580
Email: gkwpatlaw@aol.com

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia	)	
	)	
SERIAL NO.: 09/836,750	)	EXAMINER: E.C. Kemmerer, Ph.D.
	)	
FILED: April 17, 2001	)	
	)	GROUP ART UNIT: 1646
FOR: METHOD FOR GROWING MUSCLE IN A HUMAN HEART	)	

SECOND SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.

I, Andrew E. Lorincz, declare as follows:

1. I reside at 13820 NW County Rd 235, Apt 8, Alachua, FL 32616-2098.

2. This Second Supplemental Declaration is submitted in addition to my previous Declaration dated June 5, 2003 and my Supplemental Declaration dated February 3, 2004. No changes are made to either of such previous Declarations.

3. My Curriculum Vitae (hereinafter "CV") is attached as Exhibit A to my previous Declaration.

4. It is my understanding that the Examiner in charge of the above-identified patent application, in an Office Action dated June 1, 2004 for related patent application Serial No. 09/794,456, questioned my qualification, for the first time, to render my previous opinions mentioned in above Paragraph 2. It is my further understanding that the basis for such questioning was that the Examiner noted that I did not report experience with cellular therapy. I desire to provide the

information contained in following paragraph 5 so that the Examiner can consider such information in this application, as well.

5. In addition to the qualifications set forth in my CV, I am familiar with stem cell technology, including bone marrow preparation.

6. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. Such disclosures are the same as I read and understood in my previous Declaration and Supplemental Declaration. A copy of such disclosures is attached hereto as Second Supplement Declaration Exhibit A.

7. I note that the disclosures referenced in above Paragraph 6 relate to using a growth factor for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new cardiac muscle in the heart.

8. I have read and understood the claims set forth in Second Supplemental Declaration Exhibit B and have been informed that such claims are currently presented in this application.

9. Based upon above Paragraphs 6-8, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be enabled to practice the method set forth in Second Supplemental Declaration Exhibit B and to predictably anticipate the results defined therein without need for resorting to undue experimentation.

10. I believe that one skilled in the medical arts, upon reading the disclosures in above Paragraph 6, would understand that cellular growth factors, such as multifactorial and non-specific cells, are included in such disclosures. Moreover,

such skilled person would understand the disclosure on page 45 to be authored as an illustration of various modes of delivery of growth factors, whether they are genes or other genetic material; and that such skilled person would further understand that the disclosures on pages 45 and 46 describe genetic material to include appropriate cells and genes.

11. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 7-19-04

Andrew E. Lorincz, M.D.

Andrew E. Lorincz, M.D.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT A

DISCLOSURES

SECOND SUPPLEMENTAL DECLARATION
EXHIBIT A

DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or

other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in

connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the

heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT B

CLAIMS

SECOND SUPPLEMENTAL DECLARATION
EXHIBIT B

CLAIMS
APPLICATION SERIAL NO. 09/836,750

236. A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new cardiac muscle and growing a new artery in said heart.

238. The method of claim 236, further comprising repairing a dead portion of said heart.

239. The method of claim 236, further comprising repairing a damaged portion of said heart.

240. The method of claim 236, wherein said growth factor comprises genetic material selected from the group consisting of a portion of a gene, a gene, a gene product, and an extracellular matrix.

241. The method of claim 240, wherein said genetic material comprises a gene.

242. The method of claim 241, wherein said gene comprises VEGF.

243. The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.

244. The method of claim 243, wherein said growth factor comprises a cell

245. The method of claim 244, wherein said cell is multifactorial and non-specific.

246. The method of claim 245, wherein said cell comprises a stem cell.

247. The method of claim 236, wherein said growth factor is placed in said patient by injection.

248. The method of claim 247, wherein said injection is intravenous.

249. The method of claim 247, wherein said injection is intraluminal.

250. The method of claim 247, wherein said injection is intramuscular.

251. The method of claim 236, wherein said growth factor is placed in said patient by a carrier.

252. The method of claim 251, wherein said carrier comprises an angioplasty balloon.

253. The method of claim 236, wherein said growth factor comprises a gene and a cell.

254. A method of growing a new portion of a pre-existing organ comprising placing a growth factor in a body of a patient to grow new muscle in said organ.

255. The method of claim 254, wherein said organ comprises a heart.

256. The method of claim 255, wherein said new muscle comprises cardiac muscle and said growth factor comprises a stem cell.

Docket No./~~XXXXXXXX~~ 1000-10-C01

Serial No./Reg. No.: 09/836,750

Filed/~~XXXXXXXX~~ 04/17/2001

Due Date:

The Patent Office acknowledges, and has stamped hereon, the date of receipt of the items check below:

☒ Transmittal Letter	
☐ Application – Trademark	
☐ Application – Patent Specification	Total Pgs____
☐ Total Claims____ Ind. Claims ____	Total Pgs____
☐ Abstract	Total Pgs____
☐ Drawings: Formal ___ Informal ___	Total Pgs____
☐ Declaration/Oath/Power of Attorney	Total Pgs____
☐ Check No. _____	Fee: $____
☐ Assignment and Cover Sheet	Fee: $____
☐ Request for Non-Publication	
☐ Information Disclosure Statement	
☐ Form PTO-1449 w/Refs_____	Total No.
☒ Request for Extension of Time (2 mo)	Fee: $ 225.00
☒ Amendment/~~Response~~ (Exhaust-01)	
☐ Affidavit/Declaration/Statement	
☐ Brief/Reply Brief/Notice of Appeal	
☐ Fee-Base/Maintenance	Fee: $
☒ Check No. 1120	Fee: $ 700.00
☐ _____	
☐ _____	

Mailed 06/22/2006

EXHIBIT B

Third Supplemental Declaration of Dr. Lorincz

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia	)	
	)	
SERIAL NO.: 09/836,750	)	EXAMINER: Elizabeth C. Kemmerer
	)	
FILED: April 17, 2001	)	
	)	GROUP ART UNIT: 1646
FOR: METHOD FOR GROWING MUSCLE IN A HUMAN HEART	)	

THIRD SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.

I, Andrew E. Lorincz, declare as follows:

1. I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.

2. This Third Supplemental Declaration is submitted in addition to my previous Declaration, dated June 9, 2003, my Supplemental Declaration dated February 3, 2004, and my Second Supplemental Declaration dated July 19, 2004. No changes are made to any of such previous Declarations.

3. My Curriculum Vitae (hereinafter "CV") is attached as Exhibit A to my Declaration of June 9, 2003, and my background is further amplified by materials submitted in my Second Supplemental Declaration.

4. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. Such disclosures are the same as I read and understood

in my previous Declaration and Supplemental Declaration. A copy of such disclosures is attached hereto as Third Supplemental Declaration Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 37, lines 19-25; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56, line 25; and page 62, lines 1-10. A copy of such additional disclosures is attached hereto as Third Supplemental Declaration Exhibit B.

5. The disclosures in Third Supplemental Declaration Exhibit A, also contained in my previous Declaration and Supplemental Declaration, relate to using growth factors, including cells, for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new cardiac muscle. Such disclosures are also directed to the growth of new arteries in the heart.

I understand that the additional disclosures in Third Supplemental Declaration Exhibit B relate to using cellular growth factors, including bone marrow stem cells, to grow soft tissue, including an artery. Stem cells harvested from bone marrow, peripheral blood and from culture banks are described as being implanted for promoting morphogenesis and growth of all three-germ tissue layers, i.e. mesoderm, ectoderm and endoderm tissues. It would be understood by one skilled in the art that morphogenesis includes the growth of an artery, which comprises mesodermal tissue.

6. I have read and understood the claims set forth in Third Supplemental Declaration Exhibit C and have been informed that such claims will be concurrently presented in this application with this Third Supplemental Declaration.

7. Based upon above Paragraphs 4-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in the disclosures referenced therein, would be enabled to practice the method set forth in Third Supplemental Declaration Exhibit C and to predictably anticipate the results defined therein without need for resorting to undue experimentation. It is my further opinion that one skilled in the art reading such disclosures would understand that all of the well known administration procedures described at page 45 of the patent application, including intravenous, intraluminal, intramuscular, and with an angioplasty balloon, would be applicable for use in growing an artery in a human patient regardless of whether the genetic material was a gene; cell, including stem cells such as bone marrow stem cells; or another type of growth factor.

Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 5 June 2006

Andrew E. Lorincz, M.D.

Andrew E. Lorincz, M.D.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT A

DISCLOSURES

EXHIBIT A

DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell

nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles

and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT B

DISCLOSURES

EXHIBIT B

DISCLOSURES
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PAGE 33, LINES 8-10

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 40, LINE 20 – PAGE 43, LINE 3

EXAMPLE 11

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MXS-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

MXS-1 and MXS-2 transcription factors are obtained which will initiate the expression of the MXS-1 and MXS-2 homeobox genes.

The MXS-1 and MXS-2 transcription factors, BMP-2 and BMP-4 bone morphogenic proteins, and MXS-1 and MXS-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 12

Example 11 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 13

Example 11 is repeated except that the MXS-1 and MXS-2 transcription factors are not utilized. The transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

Example 13 is repeated except that the stem cells are starved and the transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 15

WT-1 and PAX genes are obtained from a sample of skin tissue is removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees.

The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factors and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The kidney germ is transplanted in the patient's body near the optic nerve. As the kidney grows, its blood supply will be derived from nearby arteries.

EXAMPLE 17

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 44, LINES 12– 13

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 48, LINES 13– 15

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

PAGE 53, LINE 1 – PAGE 56, LINE 25

EXAMPLE 18

A 36 year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F to produce an genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a

single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting

a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient cultures, physiological nutrient cultures, vectors, promotors, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials inserted ex vivo into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly timed transplantation, organ growth completes itself.

During the ex vivo application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In in vivo or ex vivo embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 19

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace in vivo a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injected intramuscularly. Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736 bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth

factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open heart surgery, endoscopic surgery, direction injection of the needle without incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both in vitro and in vivo. RAOTS external ultrasound gives a semiquantitative analysis of arterial flow. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 20

A patient, a forty year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five second increments and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 62, LINES 1–10

EXAMPLE 36

Example 18 is repeated except that the patient is a 55 year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT C

CLAIMS

EXHIBIT C

CLAIMS
APPLICATION SERIAL NO. 09/836,750

Claim 236	A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new cardiac muscle and growing a new artery in said heart.
Claim 238	The method of claim 236, further comprising repairing a dead portion of said heart.
Claim 239	The method of claim 236, further comprising repairing a damaged portion of said heart.
Claim 243	The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.
Claim 244	The method of claim 243, wherein said growth factor comprises a cell.
Claim 245	The method of claim 244, wherein said cell is multifactorial and non-specific.
Claim 246	The method of claim 245, wherein said cell comprises a stem cell.
Claim 247	The method of claim 236, wherein said growth factor is placed in said patient by injection.
Claim 248	The method of claim 247, wherein said injection is intravenous.
Claim 249	The method of claim 247, wherein said injection is intraluminal.
Claim 250	The method of claim 247, wherein said injection is intramuscular.

Claim 251 The method of claim 236, wherein said growth factor is placed in said patient by a carrier.

Claim 252 The method of claim 251, wherein said carrier comprises an angioplasty balloon.

Claim 253 The method of claim 236, wherein said growth factor comprises a gene and a cell.

Claim 257 The method of claim 236, wherein said growth factor is locally placed in said body.

Claim 258 The method of claim 238, wherein said growth factor is locally placed in said body.

Claim 259 The method of claim 239, wherein said growth factor is locally placed in said body.

Claim 260 The method of claim 243, wherein said growth factor is locally placed in said body.

Claim 261 The method of claim 236, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 262 The method of claim 238, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 263 The method of claim 239, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 264 A method of growing a new portion of a pre-existing heart comprising locally placing a growth factor comprising a stem cell in a body of a human patient to grow new cardiac muscle in said heart.

Claim 265 The method of claim 264, wherein said stem cell is placed in said patient by injection.

Claim 266 The method of claim 264, wherein said stem cell comprises living stem cells harvested from bone marrow.

Claim 267 The method of claim 266, wherein said stem cell is placed in said patient by injection.

Claim 268 The method of claim 262, wherein said stem cell is placed in said patient by injection.

Claim 269 The method of claim 263, wherein said stem cell is placed in said patient by injection.

Claim 270 The method of claim 258, wherein said growth factor comprises a cell and said cell is placed adjacent to said dead portion of said heart.

Claim 271 The method of claim 259, wherein said growth factor comprises a cell and said cell is placed adjacent to said damaged portion of said heart.

Claim 272 The method of claim 265, wherein said stem cell is injected into said heart.

Claim 273 The method of claim 267, wherein said stem cell is injected into said heart.

Claim 274 The method of claim 238, wherein said growth factor comprises a cell and said cell is placed in said body by intravenous injection.

Claim 275 The method of claim 239, wherein said growth factor comprises a cell and said cell is placed in said body by intravenous injection.

Claim 276 The method of claim 238, wherein said growth factor comprises a cell and said cell is placed in said body by intraluminal injection.

Claim 277 The method of claim 239, wherein said growth factor comprises a cell and said cell is placed in said body by intraluminal injection.

Claim 278 The method of claim 238, wherein said growth factor comprises a cell and said cell is placed in said body by an angioplasty balloon.

Claim 279 The method of claim 239, wherein said growth factor comprises a cell and said cell is placed in said body by an angioplasty balloon.

Claim 280 The method of claim 236 further comprising determining blood flow through said newly grown artery.

Claim 281 The method of claim 238 further comprising determining blood flow through said newly grown artery.

Claim 282 The method of claim 239 further comprising determining blood flow through said newly grown artery.

Claim 283 The method of claim 236 further comprising observing said newly grown artery.

Claim 284 The method of claim 238 further comprising observing said newly grown artery.

Claim 285 The method of claim 239 further comprising observing said newly grown artery.

Claim 286 A method of repairing a dead portion of a pre-existing heart comprising the steps of placing stem cells adjacent said dead portion; forming a new artery in said heart, thereby causing said dead portion of said heart to be repaired.

Claim 287 The method of claim 286, wherein said stem cells are placed by injection.

Claim 288 The method of claim 286, wherein said stem cells are placed by intraluminal administration.

Claim 289 The method of claim 286, wherein said stem cells are placed by an angioplasty balloon.

Claim 290 A method of repairing a damaged portion of a pre-existing heart comprising the steps of placing stem cells adjacent said damaged portion; forming a new artery in said heart, thereby causing said damaged portion of said heart to be repaired.

Claim 291 The method of claim 290, wherein said stem cells are placed by injection.

Claim 292 The method of claim 290, wherein said stem cells are placed by intraluminal administration.

Claim 293 The method of claim 290, wherein said stem cells are placed by an angioplasty balloon.

products, not method steps. The issue here is not whether or not workers in this technology already knew the features of the cells recited in the claims; rather, the issue is that the instant specification did not set forth contemplation of a method step wherein cells were administered intravenously, intraluminally, or via angioplasty. As discussed in the previous paragraph, the instant specification did not set forth contemplation of such method steps. The claims are being examined to the extent they read on the elected invention, administration of cells, and thus the generic concept of growth factor is not relevant. Furthermore, MPEP § 2163.02 reads:

"An Applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. See Lockwood v. American Airlines, Inc., 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention."

In the instant case, none of these criteria have been met. There was no reduction to practice, and the specification only refers to method steps involving proteins, genes and "genetic material," *but not cells*, as being useful in intravenous, intraluminal and angioplasty delivery. Therefore, the rejection is maintained.

35 U.S.C. § 112, First Paragraph – Enablement

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

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Background—Experimental data suggest that bone marrow–derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30±13 to 12±7%, $P=0.005$) and was also significantly smaller compared with the standard therapy group ($P=0.04$). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, $P=0.028$). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (*Circulation*. 2002;106:1913-1918.)

Key Words: myocardial infarction ■ cell transplantation, intracoronary ■ angiogenesis ■ bone marrow ■ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.[1,2] Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.[3]

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.[4-7] Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow–derived cells. Bone marrow contains multipotent adult stem cells that show a high capacity for differentiation.[8-10] Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.[11-14] However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.[15] Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.[16] However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

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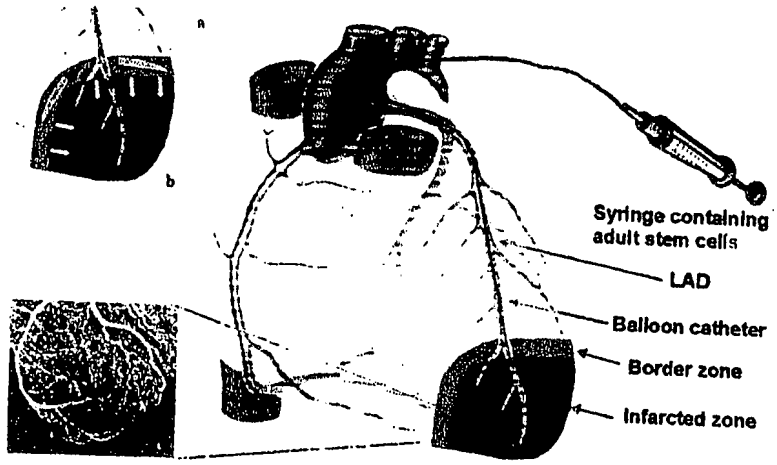

Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. a, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. b, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. c, A supply of blood flow exists within the infarcted zone.[35] The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.[17] To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery (n=4), left circumflex coronary artery (n=3), or right coronary artery (n=13). Mean duration of infarct pain was 12±10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty (n=20) and subsequent stent implantation (n=19). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β-blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (±2) days after acute coronary angiography, bone marrow (~40 mL) was aspirated under local anesthesia from ilium of cell therapy patients (n=10). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H_2O. For overnight cultivation, 1×10^6 BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was 93±3%. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10^7; this consisted of 0.65±0.4% AC133-positive cells and 2.1±0.28% CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality ex vivo control, 1×10^5 cells grown in H5100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.[18]

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.[19] All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radionuclide ventriculography, catheterization of the right heart, and

TABLE 1. Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	P
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2	...	...
Mononuclear bone marrow cells, n ($\times 10^7$)	2.8±2.2	...	...

Values are mean±SD or number of patients.
NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection ^{201}thallium scintigraphy. The contractility index P_{sys}/ESV was calculated by dividing LV systolic pressure (P_{sys}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypokinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, P=0.005). It was also significantly smaller compared with the standard therapy group after 3 months (P=0.04). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, P=0.028) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, P=NS). No significant difference was observed between the two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm^2, P=0.016, assessed by ^{201}thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

(1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m^2, P=0.010) and ejection fraction (from 51±14 to 53±13%, P=NS).
(2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, P=NS) and end-systolic volume (from 82±26 to 67±21 mL, P=0.011). Radionuclide ventriculography was used to acquire the data.
(3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, P=NS, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, P=0.005).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	*P*
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dyskinetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dyskinetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow–derived cells in experimental infarction.[11–14,18,20–23] Moreover, transendothelial migration from the coronary capillaries and incorporation of cells into heart muscle has been observed experimentally.[3,12,24–26] Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.[15,16] Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

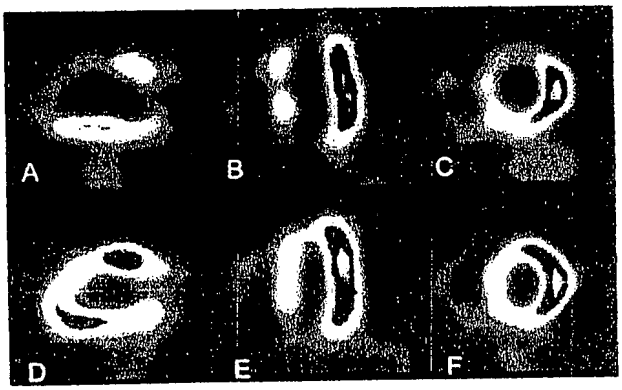

Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by ^{201}thallium scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.[27] Adult, mononuclear BMCs contain such stem and progenitor cells (≤1%), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	*P*
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume index, mL/m^2	49±7	56±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P_{syst}/ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
^{201}Thallium scintigraphy, cm^2	174±99	128±71	0.016

NS indicates not significant.

and smooth muscle cells[8-13]; (3) BMCs give rise to mesodermal progenitor cells that differentiate to endothelial cells[28]; and (4) endothelial progenitors can transdifferentiate into beating cardiomyocytes.[29] Thus, several different fractions of mononuclear BMCs may contribute to the regeneration of necrotic myocardium and vessels. In order to utilize this large and perhaps heterogeneous regenerative potential, we decided to use all mononuclear cells from the bone marrow aspirate as a whole, rather than a subpopulation. No further expansion was performed because experimental data have revealed a dramatic decline in the homing capacity of in vitro amplified hematopoietic stem or progenitor cells.[30]

The second question was how to deliver the cells most efficiently. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection: assuming a normal coronary blood flow of 80 mL/min per 100 g of LV weight, a quantity of 160 mL per left ventricle (assuming a regular LV mass of ≈200 g) will flow per minute.[31,32] This corresponds to only about 3% of cardiac output (assuming a cardiac output of 5000 mL/min).[31] Therefore, intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Thus, supplying the entire complement of cells by intracoronary administration obviously seems to be advantageous for the tissue repair of infarcted heart muscle and may also be superior to intraventricular injection,[33] because all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage. Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched with the maximum available amount of cells at all times.

As stem cells differentiate into more mature types of progenitor cells, it is thought that a special microenvironment in so-called niches regulates cell activity by providing specific combinations of cytokines and by establishing direct cellular contact. For successful long-term engraftment, at least some stem cells have to reach their niches, a process referred to as homing. Mouse experiments have shown that significant numbers of BMCs appear in liver, spleen, and bone marrow after intravenous injection.[34] To offer the BMCs the best chance of finding their niche within the myocardium, a selective intracoronary delivery route was chosen. Presumably, therefore, fewer cells were lost by extraction toward organs of secondary interest by this first pass–like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area, and the balloon was kept inflated for 2 to 3 minutes; the cells were not washed away immediately under these conditions.

The time point for delivery was chosen as 7 to 8 days after infarction onset for the following reasons:

(1) In dogs, infarcted territory becomes rich in capillaries and contains enlarged, pericyte-poor "mother vessels" and endothelial bridges 7 days after myocardial ischemia and reperfusion. Twenty-eight days later, a significant muscular vessel wall has already formed.[35] Thus, with such timing, cells may be able to reach the worst damaged parts and at the same time salvage tissue. Transendothelial cell migration may also be enhanced because an adequate muscular coat is not yet formed.

(2) Until now, only one animal study has attempted to determine the optimum time for cardiomyocyte transplantation to maximize myocardial function after LV injury. Adult rat hearts were cryoinjured and fetal rat cardiomyocytes were transplanted immediately, 2 weeks later, and 4 weeks later. The authors discussed the inflammatory process, which is strongest in the first days after infarction, as being responsible for the negative results after immediate cell transplantation, and they assumed that the best results seen after 2 weeks may have been due to transplantation before scar expansion.[36] Until now, however, no systematic experiments have been performed with BMCs to correlate the results of transplantation with the length of such a time delay.

(3) Another important variable is the inflammatory response in MI, which seems to be a superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. In the first 48 hours, debridement and formation of a fibrin-based provisional matrix predominates before a healing phase ensues.[37-40] Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the decline of adhesion molecules (intercellular adhesion molecules, vascular cell adhesion molecules) does not take place before days 3 to 4 after MI. We assumed that transplantation of mononuclear BMCs within the "hot" phase of post-MI inflammation might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels.

Taking all of this into account, we can conclude that cell transplantation within the first 5 days after acute infarction is not possible for logistical reasons and is not advisable because of the inflammatory process. On the other hand, transplantation 2 weeks after infarction scar formation seems to reduce the benefit of cell transplantation. Although the ideal time point for transplantation remains to be defined, it is most likely between days 7 and 14 after the onset of MI, as in the present study.

This trial was designed as a phase I safety and feasibility trial, meaning that no control group is necessarily required. However, to validate the results, we correlated them with those obtained from 10 patients who refused to get additional cell therapy and thus received standard therapy alone. We are aware of the fact that such a comparison does not reach the power of a randomly allocated, blinded control group. However, the significant improvement with regard to infarct region, hemodynamics (stroke volume index), cardiac geometry (LV end-systolic volume), and contractility (P_{sys}/ESV and infarction wall movement velocity) did confirm a positive effect of the additional cell therapy because the changes observed in the standard therapy group failed to reach significance.

Another important factor for interpreting the results is time interval between onset of symptoms and revascularization of the infarct-related artery by angioplasty; this represents a crucial determinant of LV recovery. For patients with acute MI, it has

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.[41] None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12±10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.[41] In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angioneogenesis and cardiomyoneogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

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Brief Rapid Communications

Bone Marrow–Derived Cardiomyocytes Are Present in Adult Human Heart

A Study of Gender-Mismatched Bone Marrow Transplantation Patients

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▸ Abstract

Background— Recent studies have identified cardiomyocytes of extracardiac origin in transplanted human hearts, but the exact origin of these myocyte progenitors is currently unknown.

Methods and Results— Hearts of female subjects (n=4) who had undergone sex-mismatched bone marrow transplantation (BMT) were recovered at autopsy and analyzed for the presence of Y chromosome–positive cardiomyocytes. Four female gender-matched BMT subjects served as controls. Fluorescence in situ hybridization (FISH) for the Y chromosome was performed on paraffin-embedded sections to identify cells of bone marrow origin with concomitant immunofluorescent labeling for -sarcomeric actin to identify cardiomyocytes. A total of 160 000 cardiomyocyte nuclei were analyzed approximating 20 000 nuclei per patient. The mean percentage of Y chromosome–positive cardiomyocytes in patients with sex-mismatched BMT was 0.23±0.06%. Not a single Y chromosome–positive cardiomyocyte was identified in any of the control patients. Immunofluorescent costaining for laminin and chromosomal ploidy analysis with FISH showed no evidence of either pseudonuclei or cell fusion in any of the chimeric cardiac myocytes identified.

Conclusions— These data establish for the first time human bone marrow as a source of extracardiac progenitor cells capable of de novo cardiomyocyte formation.

Methods
Results
Discussion
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Key Words: chimera • stem cells • myocytes, cardiac • transplantation, bone marrow

Introduction

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The concept of the human heart as an organ incapable of self-renewal has recently been challenged by identification of cardiac myocytes of probable extracardiac origin in hearts of patients undergoing sex-mismatched cardiac transplantation.[1–4] The exact source of these cells is currently unclear, but data from experiments in animals support a bone marrow origin.[5] It is important to note that a marked discrepancy in the level of cardiac chimerism has been observed in the gender-mismatched cardiac transplantation setting.[1–4,6,7] Moreover, controversy has arisen with regard to the methodologies used to define chimeric cardiac myocytes in these human studies. Specifically, concerns have recently been raised about the most appropriate techniques required to differentiate (1) true cardiac myocyte nuclei from pseudonuclei,[6] and (2) diploid nuclei from epigenetic phenomena, such as spontaneous cell fusion.[8]

To address the above issues, we used a specific study design and experimental approach. An ideal method to answer the question of bone marrow origin of chimeric myocytes is to analyze hearts of patients who have undergone gender-mismatched bone marrow transplantation (BMT). The presence of Y chromosome–positive cardiomyocytes in the hearts of female patients would conclusively suggest a bone marrow origin for these cells. By using fluorescence in situ hybridization (FISH) combined with

immunohistochemistry, we determined the percentage of male cardiomyocytes in autopsy hearts of female patients who had undergone gender-mismatched BMT. To exclude the possibility of false identification of pseudo or fusion nuclei as chimeric cardiomyocytes, additional analyses were performed with the use of basement membrane laminin costaining and chromosome 18 multiploidy analysis with FISH, respectively. Gender-matched BMT patients served as controls.

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Patients and Autopsy Tissue

A review of the Mayo Clinic BMT database and autopsy records identified 4 female patients who had received male donor bone marrow. Female patients who had gender-matched BMT were examined as controls. The Mayo Clinic institutional review board granted approval for the study of human tissue samples.

Combined Immunohistochemical and FISH Analysis

Immunohistochemical analysis of cardiac tissue sections was performed by using a monoclonal antibody against -sarcomeric actin (Sigma clone 5c5) and a rabbit antibody against laminin (Sigma, St Louis, Mo). The secondary detection used was respectively an anti-mouse antibody conjugated to Cy-3 (Molecular Probes; red) and an anti-rabbit antibody conjugated to Alexa Fluor (Molecular Probes; green). In separate experiments, liver and skeletal muscle tissue from the same subjects was stained with antibodies to human hepatocyte and skeletal muscle actin with the use of monoclonal antibodies (both from Dako). Hepatocytes and skeletal myocytes were visualized using a secondary anti-mouse antibody conjugated to Cy-3.

After immunostaining, FISH was immediately performed as previously described.[3] The X and Y chromosome (CEP X, Y; Vysis Inc; B7322, B-6927) DNA probes used were specific for the satellite region of each chromosome and labeled with Cy-3 and fluorescein isothiocyanate, respectively. For combined analysis, sarcomeric actin and laminin staining and FISH for Y-chromosome were used. In separate experiments a probe to the centromere of human chromosome 18 (CEP 18 Aqua; light blue dot; Vysis) was combined with X (red dot) and Y chromosome (green dot) analysis to evaluate cell ploidy and exclude cell fusion in the chimeric nuclei identified.

In all cases, FISH signals were enumerated using a Zeiss Axioplan microscope equipped with a triple-pass filter (Vysis). Rigorous criteria were used to identify Y chromosome–positive cardiac myocytes as previously described.[2] Counting of the nuclei and Y chromosome was performed by two independent blinded observers.

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Patient Characteristics

The clinical profiles of the 4 female patients who underwent sex-mismatched BMT are shown in the Table. Subjects had a range of hematologic diseases requiring BMT ($2.8\pm0.5\times10^8$ infused cells/kg body weight) and received the same pretransplantation conditioning regimen, which consisted of total body irradiation and cyclophosphamide. All patients were maintained on prednisone, and 2 subjects were maintained on additional cyclosporine and azathioprine after transplantation. Autopsy examination showed no macroscopic or microscopic evidence of inflammation in any of the hearts studied (Figure, A).

View this table: **Clinical Data and Cardiomyocyte Chimerism Analysis of Gender-Mismatched BMT Patients**
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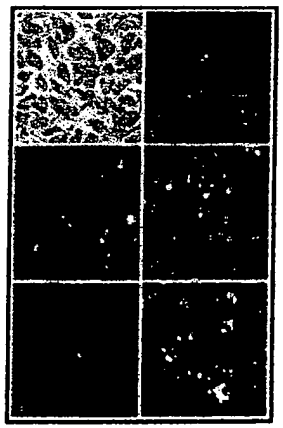

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A, Hematoxylin-and-eosin staining of normal left ventricular myocytes showing no evidence of inflammatory cell infiltrate. B, Cardiomyocyte of female gender-mismatched BMT patient staining positive for α-sarcomeric actin (red) possessing nuclei (blue) positive for Y chromosome (green dot). B, inset, Diploid bone marrow–derived cardiomyocyte nucleus of female gender-mismatched BMT patient showing X chromosome (open arrowhead, red dot), Y chromosome (green dot), and a pair of chromosome 18 (filled arrows, light blue dots) signals; note overlying and surrounding red staining for α-sarcomeric actin. C, Y chromosome–positive true nucleus (blue, green dot; arrow) of bone marrow–derived cardiomyocyte cytoplasm (sarcomeric actin, red) surrounded by basement membrane laminin (green, arrowhead). D, Y chromosome–positive pseudonucleus (open arrowhead) separated from cardiomyocyte (sarcomeric actin, red) by laminin (green-filled arrowheads). E and F, Combined immunofluorescence staining and FISH for Y chromosome in female gender-mismatched BMT subjects showing (E) male skeletal muscle cell (red cytoplasm and blue nucleus with green dot-arrow) and (F) male hepatocyte (red cytoplasm and blue nucleus with green dot-arrow). Note a male cell (open arrowhead) that does not costain with hepatocyte antibody.

Immunofluorescence and FISH Analysis

Histological sections of the left ventricle in gender-mismatched subjects revealed a mean percentage of Y chromosome–positive cardiac myocytes of 0.23±0.06% (Table). The Y chromosome was located eccentrically within the nuclei of chimeric cardiomyocytes (Figure, B and C), and chromosomal ploidy analysis excluded cell fusion (Figure, B, inset). Four female control patients who had undergone sex-matched BMT showed no evidence of Y chromosome positivity in any of the 80 000 cardiomyocyte nuclei analyzed. Basement membrane laminin and sarcomeric actin costaining distinguished true chimeric nuclei with surrounding myocyte cytoplasm from pseudonuclei (Figure, C and D). Male bone marrow–derived hepatocytes and skeletal myocytes were also found in the liver and muscle of female gender-mismatched BMT recipients (Figure, E and F), and mean donor cell chimerism in these tissues was 0.4% and 0.2%, respectively (3000 nuclei analyzed). The detection sensitivity of FISH for Y chromosome in this study was 45%, similar to that cited in previous FISH analysis of tissue sections.[2,4]

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These data suggest that adult human bone marrow acts as a source of extracardiac progenitor cells contributing to cardiomyocyte formation. The additional use of laminin costaining and chromosomal ploidy analysis in this study makes the possibility of confusing pseudonuclei or cell fusion events for chimeric myocytes unlikely. The potential origin and phenotype of marrow myocyte precursors in our subjects includes lineage-restricted mesenchymal,[9] hematopoietic,[10] and multipotent adult progenitors[9] and cells of angioblastic lineage.[11]

Physiological stress and tissue injury are known to release cytokines and chemokines, which may promote mobilization of progenitor cells from the bone marrow to the peripheral circulation.[12] Although no patients in our study group had histological evidence of myocardial inflammation, 3 of 4 patients had respiratory complications such as adult respiratory distress syndrome and bronchiolitis obliterans. It is possible that severe tissue injury occurring in these conditions resulted in high levels of circulating cytokines with consequent mobilization of circulating progenitor cells. Interestingly, prior animal experiments showed no detectable engraftment of marrow-derived cells in the absence of myocardial injury.[5] The difference between these animal data and our study may reflect differences in species, duration of study, use of "side population" cells exclusively versus unfractionated bone marrow, or other poorly understood phenomena associated with clinical disease and its treatment.

The consistent levels of chimerism seen at 5 weeks and 20 months after marrow transplantation in our present study suggest a steady-state recruitment of marrow progenitors rather than an initial seeding event early after transplantation. It is noteworthy that a similar

recruitment of bone marrow cells occurred in the liver and skeletal muscle as well as the heart, which validates previous animal and human data suggesting multipotent differentiation potential for bone marrow–derived cells.[11,13] It is well known that marrow-derived mesenchymal and hematopoietic stem cells circulate for long periods after transplantation, allowing an equilibrium to be established between circulating and tissue-specific seeding compartments. It is therefore conceivable that low-level recruitment of blood-borne precursors into the myocardium occurs in response to local events in the tissue microenvironment.

Another possibility is that myocardial injury secondary to the pretransplantation conditioning regimen leads by a repair response to recruitment of marrow precursors into the myocardium. This scenario seems less likely, however, as the degree of chimerism would be expected to decrease over time and a concurrent "response to injury" would be expected from other blood-borne cells such as leukocytes, neither of which was seen in our study. Furthermore, because all our patients had established hematologic disease before BMT, we cannot automatically infer that chimeric events seen in our study occur under normal healthy conditions, nor can we exclude the possibility that pretransplantation disease may have altered posttransplantation seeding of circulating cells. Finally, we can only speculate on the additional modulating effects of immunosuppressive therapy on bone marrow cell recruitment in our subjects.

The mean percentage of bone marrow–derived cardiac myocytes observed in our subjects was low. It is difficult if not impossible to compare our data with previous chimerism studies both from a clinical and methodological perspective[1–4,7] because it is likely that variables such as chimeric cell detection method, time of study after transplantation, and the presence or absence of inflammation influence the level of myocyte chimerism observed. Finally, while this manuscript was under review, Thiele et al[14] reported 6.4% cardiomyocyte chimerism in a group of male bone marrow transplantation patients, a level more than an order of magnitude greater than our findings. However, the small number of nuclei analyzed and the use of morphology instead of myocyte-specific staining make the identification of chimeric nuclei as true cardiomyocytes less certain in this study.

In conclusion, the present study establishes bone marrow as a contributor to low-level de novo cardiac myocyte formation. The clinical significance of this finding in terms of myocardial regeneration will depend on the success of future efforts to understand and augment the mobilization, homing, and differentiation properties of these cells. Further investigation may also determine whether these cells can be engineered or targeted to diseased myocardium for therapeutic effect.

Acknowledgments

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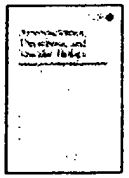

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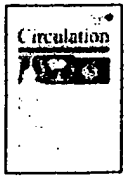

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Skeletal Myoblast Transplantation for Repair of Myocardial Necrosis

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Abstract

Myocardial infarcts heal by scarring because myocardium cannot regenerate. To determine if skeletal myoblasts could establish new contractile tissue, hearts of adult inbred rats were injured by freeze-thaw, and 3–4.5 × 10^6 neonatal skeletal muscle cells were transplanted immediately thereafter. At 1 d the graft cells were proliferating and did not express myosin heavy chain (MHC). By 3 d, multinucleated myotubes were present which expressed both embryonic and fast fiber MHCs. At 2 wk, electron microscopy demonstrated possible satellite stem cells. By 7 wk the grafts began expressing β-MHC, a hallmark of the slow fiber phenotype; coexpression of embryonic, fast, and β-MHC continued through 3 mo. Transplanting myoblasts 1 wk after injury yielded comparable results, except that grafts expressed β-MHC sooner (by 2 wk). Grafts never expressed cardiac-specific MHC-α. Wounds containing 2-wk-old myoblast grafts contracted when stimulated ex vivo, and high frequency stimulation induced tetanus. Furthermore, the grafts could perform a cardiac-like duty cycle, alternating tetanus and relaxation, for at least 6 min. Thus, skeletal myoblasts can establish new muscle tissue when grafted into injured hearts, and this muscle can contract when stimulated electrically. Because the grafts convert to fatigue-resistant, slow twitch fibers, this new muscle may be suited to a cardiac work load. (*J. Clin. Invest.* 1996. 98:2512–2523.) Key words: myocardial infarction • skeletal myoblast • myosin heavy chain • contractile function • cell transplantation

Introduction

Experimental and clinical therapies for myocardial infarction have focused traditionally on limiting infarct size. Unfortunately, the goal of limiting myocardial injury has been difficult to achieve clinically, because ischemic myocardium dies quite rapidly (1) and most patients wait more than 3 h after coronary occlusion before seeking medical attention. As an alternative approach, we are exploring strategies to induce the injured heart to heal with muscle replacement rather than forming scar tissue.

One strategy for muscle regeneration is to transplant either skeletal or cardiac myocytes into the injured heart. Studies from Field's group showed that cardiac myocytes can be transplanted into normal hearts, where they couple with host cardiocytes via intercalated discs (2, 3). However, a major drawback to using cardiocytes is their inability to proliferate in culture. At present it seems unlikely that enough primary cardiocytes could be obtained from the patient or histocompatible donor to repair a myocardial infarct in humans. On the other hand, skeletal muscle satellite cells (muscle stem cells) proliferate well in culture. Satellite cells could be obtained from muscles of infarct patients and rapidly expanded in culture, or stocks of potentially therapeutic myoblasts could be obtained from embryos and frozen for subsequent use (4, 5). Furthermore, physiological studies have shown that when properly conditioned, skeletal muscle can adapt to perform a cardiac-type work load (6). Recent studies have demonstrated the feasibility of grafting skeletal myoblast lines into normal hearts (7) and autologous satellite cells into injured hearts (8, 9). However, to generate significant amounts of functional new muscle the transplanted cells ideally should proliferate and then differentiate into mature myofibers capable of sustaining a cardiac work load. This study was performed to determine the proliferation and differentiation patterns of skeletal myoblasts after engraftment into injured rat hearts and to determine whether this new muscle could support contractile activity.

Methods

Skeletal myoblast isolation and culture. These studies were approved by the University of Washington Animal Care Committee and were conducted in accordance with federal guidelines. Skeletal myoblasts were obtained from the limbs of 1–3-d-old Fischer rats. This inbred strain was used to avoid immune barriers to transplantation. After time of killing, the carcasses were skinned and the limbs were placed into cold tissue culture media. Under a dissecting microscope, the muscles were stripped of surrounding adipose tissue and fascia and bluntly dissected from their tendons. The muscles were minced with iridectomy scissors until a fine slurry was formed. The slurry was then digested in 0.05% trypsin/EDTA (GIBCO-BRL, Gaithersburg, MD) in Ham's saline A at 37°C, with intermittent mechanical agitation to assist dispersal. After 30–45 min the cell suspension was filtered through sterile gauze to remove undispersed tissue fragments and rod shaped mature myofibers. Cells were plated at ~ 5 × 10^6 cells/dish in 100-mm gelatinized plates in 10 ml Ham's F10C media, containing 15% horse serum and 50 μg/ml gentamicin sulfate (Gemini Bioproducts, Inc., Calabasas, CA). Recombinant human basic fibroblast growth factor was added twice daily to a final concentration of 6 ng/ml, and the complete medium was replaced once per day. Approximately 10% of the cells attached and grew with a doubling time of ~ 18 h. The cultures contained a mix of small, oval myoblasts and elongated, spindle-shaped cells consistent with fibroblasts. Subconfluent cultures were passaged every 2–3 d (1:5 split) to minimize the occurrence of myogenic differentiation at higher density. On the day before transplantation, the cultures were tagged for subsequent identification in vivo. In some experiments cells were tagged with fluorescent micro-

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spheres (1:500 dilution of stock 200 nm yellow-green fluorescent microspheres; Molecular Probes, Eugene, OR). The latex microspheres were endocytosed (typically > 20 spheres/cell) and served as cytoplasmic markers (10). In other experiments, cells were incubated overnight with [^{3}H]thymidine (1 μCi/ml) to mark their nuclei after autoradiography. Cultures were trypsinized immediately before transplantation and suspended at a concentration of $\sim 3 \times 10^7$/ml. Small aliquots of the remaining cell suspension were replated at $\sim 2 \times 10^4$ cells/cm^2 into gelatinized, multichamber plastic slides, and fixed in methanol after various culture intervals for immunostaining.

Rat cardiac injury models. Inbred male Fischer rats (Simonsen Labs, Gilroy, CA) weighing 350–400 grams were anesthetized with intraperitoneal ketamine-xylazine (68 and 4.4 mg/kg, respectively), intubated, and mechanically ventilated with room air. The heart was exposed aseptically via a left thoracotomy, and a 1-cm-diameter aluminum rod, precooled with liquid nitrogen, was placed in direct contact with the anterior left ventricle for 15 s. Freeze-thaw reproducibly caused a disc-shaped region of coagulation necrosis, $\sim$ 1 cm in diameter, extending $\sim$ 2 mm into the myocardium. It should be noted that while infarcts typically have irregular borders with viable peninsulas of subepicardial myocardium along penetrating vessels, freeze-thaw lesions consist of confluent necrosis in the subepicardium with viable myocardium in the subendocardium. Because they are not transmural, freeze-thaw lesions do not cause cardiac aneurysm formation. Despite these differences, the cellular patterns of coagulation necrosis, inflammation and phagocytosis, granulation tissue formation, and scarring after freeze-thaw injury are indistinguishable from myocardial infarction (11–13), making it a suitable model to study myocardial repair.

In the initial studies, $\sim 3 \times 10^6$ myoblasts in 100 μl tissue culture media were injected superficially into the center of the injured region immediately after injury, using a 27-gauge needle. Then, the chest was closed and the rats were allowed to recover for timed intervals from 1 d to 3 mo (n = 4/time point). To mimic a clinical situation more closely, a second protocol was used in which the freeze-thaw lesion was allowed to heal for 1 wk before transplanting myoblasts. By 1 wk, most of the necrotic myocardium had been replaced by granulation tissue, but scar formation had not yet begun. The rats (n = 2/time point; no 3 d or 3 mo time points) were reanesthetized and a thoracotomy was repeated. The heart was exposed and a 100-μl suspension containing $\sim 3 \times 10^6$ myoblasts was injected into the wound as described above. The chest was closed and the animals were allowed to recover for intervals from 1 d to 7 wk.

To detect DNA synthesis in the grafts the rats were treated with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU)[1] (Boehringer-Mannheim, Indianapolis, IN). 1 d before time of killing, the rats were lightly anesthetized, and a 50-mg tablet of BrdU was implanted subcutaneously for measurement of cell replication. Preliminary studies showed that a subcutaneous 50-mg BrdU tablet gave comparable replication rates to a 24-h continuous infusion with an osmotic mini-pump (not shown). For rats killed 1 d after transplantation, a single 10-mg pulse of BrdU was given intraperitoneally 1 h before time of killing. This avoided incorporation of BrdU into the cells which were cycling at the time of transplantation.

Rats were killed with a pentobarbital overdose and their hearts were excised. In the immediate transplantation groups, the aorta was cannulated and the hearts were perfusion fixed with methyl Carnoy's solution (60% methanol, 30% chloroform, 10% glacial acetic acid), transversely sectioned, and embedded in paraffin by routine methods. In groups transplanted 1 wk after injury, the hearts were transversely sectioned, embedded in OCT (Miles Inc., Kankakee, IL), and frozen in a dry ice–ethanol bath for frozen section analysis. In both protocols, sections of gut were obtained as controls for measurement of cell replication with BrdU.

1. *Abbreviations used in this paper:* BrdU, 5-bromo-2'deoxyuridine; MHC, myosin heavy chain.

Measurement of contractile function in isolated wound strips. Rat hearts were given 4.5×10^6 myoblasts (n = 8) in 100 μl or a sham injection of saline (n = 3) immediately after injury. 2 wk after engrafting, the hearts were excised and transversely sectioned. Under a dissecting microscope, most of the subendocardial myocardium was trimmed away from the injured region, and isolated wound strips ($\sim 1.5 \times 1.5 \times 8$ mm) were prepared. One or two strips were studied from each myoblast-engrafted heart, and two or three strips were studied from each sham-injected heart. The strips were ligated at both ends with silk suture and then placed in a bath of physiological saline with the following composition (mmol/liter): 116 NaCl, 4.6 KCl, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 26 Mops (pH 7.4), 11 glucose, and 10 mg/liter gentamicin. The buffer was equilibrated with 95% O_2/5% CO_2 and maintained at 20°C via a thermostatically controlled water jacket. Wound strips were mounted between an isometric force transducer (model 60-2995; Harvard Apparatus, Inc., South Natick, MA) and a fixed glass hook. Resting tension was set initially at 0.5 g. Strips were stimulated with 1-ms bipolar pulses delivered via platinum wire electrodes using a Grass model S48 stimulator (Astro-Med, Inc., West Warwick, RI). Voltage was increased in 10-V increments until contractile activity was observed. Force traces were displayed on a digital storage oscilloscope (model 3091; Nicolet Instrument Corp., Madison, WI) and recorded using a General Scanning model RS4-5P strip chart recorder. After determining the force–voltage relationship, the optimal length for force production was determined for each wound strip using test contractions at 2-min intervals, a time sufficient for metabolic recovery in mammalian fast twitch muscles (14). Force–frequency analysis was performed by increasing the stimulation frequency in 1-Hz increments; tetanus was defined as the point where the oscillations of contractile force at the plateau were < 3% of the net force generated (14). Finally, to test fatigability the grafts were subjected to a simulated cardiac-like duty cycle, consisting of 0.33 s of tetanus followed by 0.67 s of relaxation (1:2 cycle), continuing for 6 min. After completion of functional studies the strip's cross-sectional area was determined, and the tissue then was processed for histology or electron microscopy.

Immunocytochemistry. Antibodies used for immunostaining are given in Table I. 6-μm frozen sections were cut on a cryostat, briefly air dried, and stored at −70°C until use. 5-μm paraffin sections were deparaffinized in xylene and rehydrated in a graded alcohol series. Cultured cells were fixed and stored in cold PBS until use. For all samples, endogenous peroxidase activity was quenched by incubating with 0.3% H_2O_2 in methanol for 30 min. Immunostaining was carried out at room temperature. Sections were blocked with 1.5% normal horse serum in PBS for 1 h. The sections were then incubated with the primary antibody in 1.5% horse serum for 1 h, followed by incubation with the secondary antibody (rat adsorbed horse anti-mouse, 1:400 dilution; Vector Labs, Inc., Burlingame, CA) for 1 h. Antigens were localized with an avidin-biotin-peroxidase complex (ABC Elite kit; Vector Labs). For staining with a single antibody, diaminobenzidine (Sigma Immunochemicals, St. Louis, MO) was used as a chromagenic substrate. For double immunolabeling with antibodies to myosin and BrdU, sections were first exposed to 1.5 N HCl for 15 min at 37°C to denature the DNA, followed by a rinse in 0.1 mol/liter borax to stabilize the denatured strands. Sections were then stained routinely for myosin heavy chain (MHC) using diaminobenzidine. After a second quenching in 0.3% H_2O_2, sections were blocked with 1.5% normal horse serum, and then incubated with a mouse monoclonal antibody to BrdU for 1 h. After incubation with the secondary antibody (horse anti-mouse), BrdU was localized with an avidin-biotin-peroxidase complex, using True Blue (KPL, Gaithersburg, MD) as substrate. Cross-reactivity between the first primary antibody and the second secondary antibody did not occur, as long as the True Blue substrate was incubated for a short duration (< 1 min). Sections were counterstained either with methyl green, nuclear fast red, or hematoxylin.

Electron microscopy. After measurement of contractile function, one of the tissue strips was immersed in half strength Karnovsky's fix-

Table I. Antibodies Used for Immunocytochemistry

Antibody	Antigen recognized	Dilution	Source	Reference
MF-20	Sarcomeric MHCs	Hyb. Sup., 1:100	American Type Culture Collection, Rockville, MD	39
MY-32	Skeletal MHC-fast (types IIA and IIB)	Mouse ascites, 1:2000	Sigma Immunochemicals	40
BA-G5	Cardiac MHC-α	Hyb. Sup., 1:5	American Type Culture Collection	41
F1.652	Embryonic MHC	Hyb. Sup., 1:100	Developmental Studies Hybridoma Bank*	42
A4.951	β-MHC	Hyb. Sup., 1:50	American Type Culture Collection	43
Anti-BrdU	BrdU	IgG, 1:50000	Eurodiagnostics, Apeldoorn, The Netherlands	44

IgG, purified IgG monoclonal antibody; *Hyb. Sup.*, hybridoma supernatant. * The monoclonal antibody F1.652, developed in the laboratory of Dr. Helen Blau, was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract NO1-HD-2-3144 from the National Institute of Child Health and Human Development.

ative and dissected into small cubes < 1 mm in greatest dimension. The tissue was fixed overnight in half strength Karnovsky's fixative, postfixed for 1 h in 1% osmium tetroxide at room temperature, dehydrated through a graded alcohol series followed by propylene oxide, and embedded in Medcast resin (Ted Pella, Inc., Redding, CA). Semithin sections were stained with toluidine blue and examined by light microscopy. Thin sections were cut from selected blocks, stained with lead citrate and uranyl acetate, and examined in a Jeol JEM 1200EXII transmission electron microscope. Representative areas were photographed.

Results

Characteristics of myoblast cultures. The muscle cultures contained a mixed cell population. At least 22% of the cells were skeletal muscle, as indicated by their staining for sarcomeric myosin after switching to a differentiation medium containing 1.5% serum and no FGF for 3 d. This procedure underestimates the true percentage of skeletal muscle cells by severalfold, since the nonmyogenic cells continue to divide after the medium switch while the myoblasts complete their present cell cycle and then terminally differentiate. Approximately 1% of the cells stained with antibodies to smooth muscle α-actin, which can mark either smooth muscle cells or fibroblasts. Virtually none of the cells stained with an antibody for the endothelial marker von Willebrand factor. The remaining cells were presumably fibroblasts.

Histology and differentiation patterns of myoblast grafts. Cultured skeletal myoblasts were transplanted into cardiac freeze-thaw lesions either immediately after injury, or, to mimic a clinical situation more closely, cells were transplanted 1 wk after injury. The two protocols yielded similar results and will be described together; minor differences are noted below. On the first day after transplantation the myoblasts were mononuclear cells (Fig. 1 *A*). The grafted cells could be distinguished clearly from inflammatory cells within the necrotic tissue by their larger size and characteristic oval shape. (Fibroblast ingrowth from the surrounding tissue had not yet begun at this time.) The identity of the grafted cells was confirmed by their cytoplasmic fluorescent microspheres and radioactive nuclei (not shown). Mitotic figures were common. The grafted cells did not stain with antibodies to skeletal or cardiac MHCs (Fig. 1 *B*). Thus, muscle differentiation had not yet occurred.

By 3 d after transplantation, many of the grafted cells had fused to form multinucleated myotubes (Fig. 1 *C*). Myotubes were partially aligned along the short (transverse) axis of the heart. The myotubes stained with antibodies to sarcomeric MHC, embryonic MHC (Fig. 1 *D*), and to MHC-fast (not shown). Occasional cross-striations were noted, but these were not frequent at this time (Fig. 1 *D*). The myotubes did not express cardiac MHC-α. By 1 wk the grafts were easily recognizable as skeletal myofibers and many cells contained cross-striations. As before, the new myofibers stained with antibodies to sarcomeric MHC, embryonic MHC, and MHC-fast, but did not express cardiac MHC-α (not shown). By 2 wk after transplantation the grafts had the appearance of maturing skeletal myofibers (Fig. 1 *E*). Sarcomeres were well formed, and many cells had peripheral nuclei. The myofibers stained intensely with antibodies to sarcomeric myosin, embryonic MHC (Fig. 1 *F*), and skeletal MHC-fast (Fig. 1 *G*). No staining with cardiac MHC-α antibodies was observed at 2 wk.

At 7 wk after transplantation the grafts were islands of mature skeletal muscle within young scar tissue (Fig. 1, *H–J*). There was a moderate increase in cell diameter compared with 2 wk. None of the muscle grafts were infiltrated or splayed apart by scar tissue, nor was there evidence of fiber atrophy. Vascular density appeared normal for muscle tissue (Fig. 1 *J*). All of the 7-wk grafts stained strongly with antibodies to sarcomeric myosin and embryonic MHC (Fig. 1 *H*). The grafts injected immediately after injury stained intensely with antiskeletal MHC-fast, comparable with Fig. 1 *F*. In contrast, the grafts injected 1 wk after injury stained poorly with antiskeletal MHC-fast (see below). No staining with antibodies to cardiac MHC-α was observed in the grafts, while the adjacent myocardium stained intensely (Fig. 1 *I*).

At 3 mo after transplantation the grafts again had the appearance of mature skeletal muscle (Fig. 1 *K*). Most myofibers had peripheral nuclei, and vascular density appeared normal. Fiber diameter was generally larger than in the 7-wk group, indicating that the cells had hypertrophied between 7 wk and 3 mo (compare Fig. 1, *J* and *K*). In one heart, however, part of the graft was infiltrated by scar tissue which encircled individual myofibers and was associated with fiber atrophy (Fig. 1 *L*). The grafts continued to express embryonic MHC and MHC-fast (not shown). Once again, no staining with antibodies to cardiac MHC-α was observed (comparable with Fig. 1 *I*). At all time points the myofibers were predominantly aligned parallel with the short (transverse) axis of the heart and therefore appeared in longitudinal section. However, some fascicles of muscle appeared obliquely or cross-sectioned in this plane.

In summary, the grafts began to differentiate into myo-

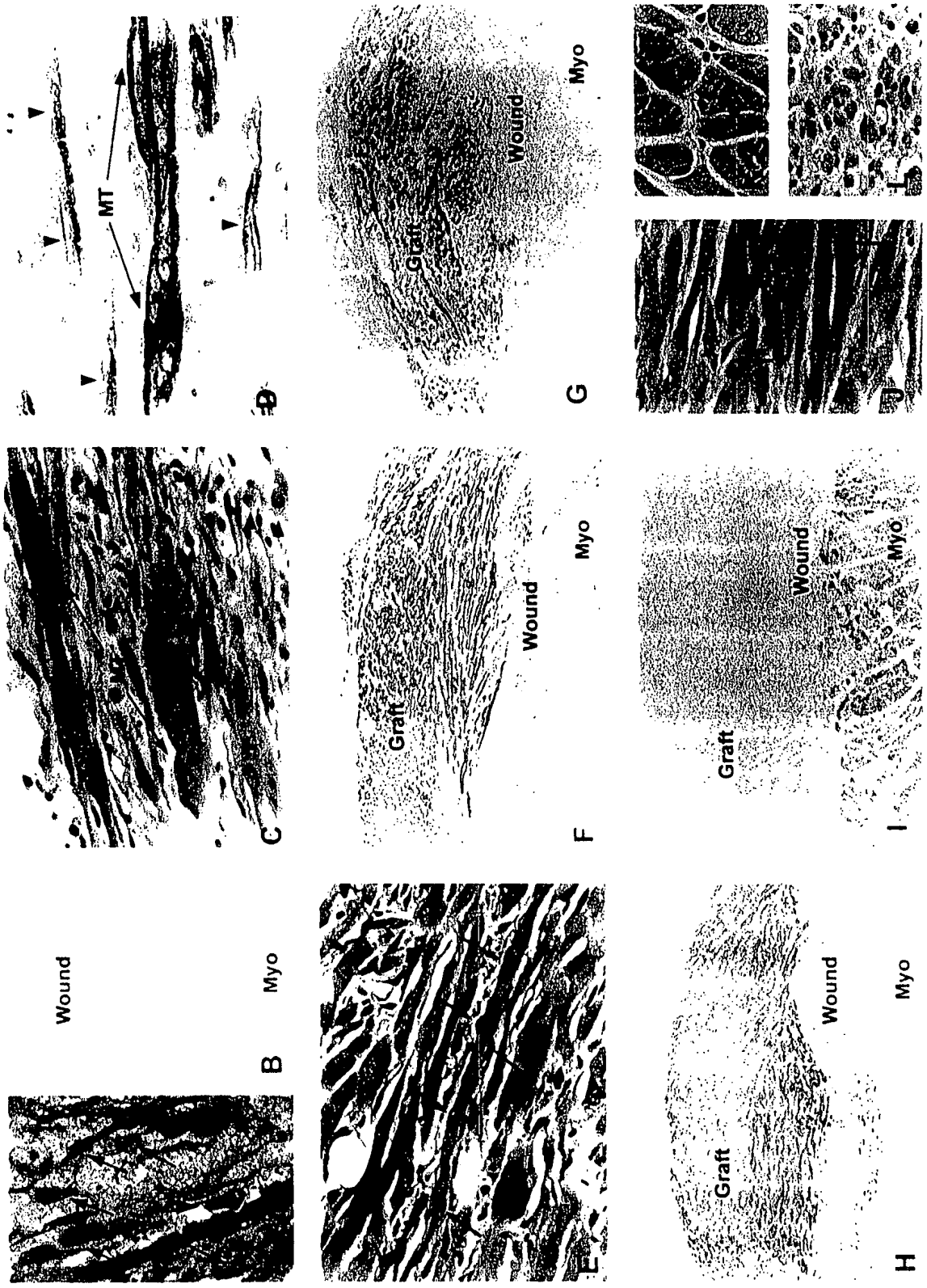

Figure 1. Morphology and MHC expression in skeletal myoblast grafts. Rat hearts were injured by freeze-thaw and syngeneic skeletal muscle cells were grafted into the lesions. All panels in this figure are from hearts which were grafted immediately after injury. (*A*) 1-d graft. The grafted cells are identifiable as relatively large, oval shaped cells (*arrows*) within the necrotic myocardium. One graft cell is in mitosis (*arrowhead*). Numerous smaller inflammatory cells are present within the lesion. Fibroblast ingrowth from surrounding viable tissue had not yet begun at this

tubes between 1 and 3 d and acquired the appearance of maturing myofibers with well formed sarcomeres by 2 wk. The grafts expressed both embryonic MHC and MHC-fast at all times between 3 d and 3 mo. There was no expression of cardiac MHC-α at any time.

Electron microscopy. Electron microscopy was performed on one heart, 2 wk after myoblast grafting. Most of the grafted cells had well formed, slightly contracted sarcomeres which were aligned in registry (Fig. 2 *A*). Mitochondria were abundant. Multinucleation was evident in many cells, as were well formed T-tubules. However, in other cells a spectrum of morphological stages was present, consistent with developing skeletal muscle (Fig. 2 *B*). Cells at the earliest stage were small, had scanty myofibril content, and contained focal aggregations of electron-dense material suggestive of developing Z-discs. In these cells there were abundant ribosomes and glycogen, a prominent Golgi apparatus, and dilated segments of sarcoplasmic reticulum. Intermediate cells were larger and had increasing amounts of myofibrils with a corresponding decrease in ribosomes and glycogen. Some cells had well formed sarcomeres, but these were out of registry compared with the most mature cells. No intercalated discs were identified between cells in the graft region. Adjacent myofibers often had intimately apposed, interdigitating cell membranes. Occasional cells were identified with electron-dense membrane structures suggestive of intermediate adherens junctions and gap junctions (Fig. 2, *C* and *D*). Some mature myofibers were closely associated with mesenchymal cells, located within the basal lamina compartment of the myofiber. Their location within the basal lamina of the myofiber suggests that they might be new satellite stem cells (Fig. 2, *E* and *F*). Some of these mesenchymal cells had abundant rough endoplasmic reticulum, similar to fibroblasts. Cells with this morphology have also been described in regenerating skeletal muscle by Trupin et al. (15). Their location within the basal lamina of the myofiber and the absence of collagen in this space make it unlikely that these cells are actually fibroblasts.

Myoblast grafts convert from fast to slow twitch fibers. The poor staining for MHC-fast in the 7-wk group with delayed transplantation seemed at variance with the morphology of the grafts, which showed relatively hypertrophic cells with well formed sarcomeres. We hypothesized that the grafts had undergone fiber type conversion to slow twitch muscles, which no longer expressed high levels of MHC-fast. Slow twitch fibers have physiological similarities to cardiac muscle, including a high capacity for oxidative phosphorylation and fatigue resistance. Furthermore, slow fibers use β-MHC as a major contractile protein, which is also the predominant myosin in developing rat hearts. In contrast, fast twitch fibers use glycolysis for ATP production, have a low aerobic capacity and fatigue rapidly, and do not express β-MHC (16). Therefore, we compared β-MHC expression with skeletal MHC-fast, to determine fiber types in the maturing grafts.

At 1 wk the grafts stained intensely for MHC-fast (Fig. 3 *A*) but did not stain with an antibody to β-MHC (Fig. 3 *B*). At 2 wk the grafts continued to express MHC-fast. In the group transplanted immediately after injury no expression of β-MHC was noted at 2 wk, yet in grafts transplanted 1 wk after injury some cells expressed β-MHC (not shown). At 7 wk after transplantation the two groups differed in expression of MHC-fast, with strong staining in the immediate transplant group (see Fig. 1 *G*) and weak staining in the group where transplantation was delayed for 1 wk after injury (Fig. 3 *C*). However, both the immediate and delayed transplantation groups exhibited extensive staining for β-MHC at 7 wk after transplantation (Fig. 3 *D*). At 3 mo there was continued expression of β-MHC and MHC-fast in the immediate transplantation group; we did not study the delayed transplantation protocol at 3 mo. Thus, myoblast grafts appeared to be undergoing conversion from fast twitch to slow twitch fibers. Conversion appeared to take place

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time. Hematoxylin and eosin stain. ×800. (*B*) Low magnification of 1-d graft stained for embryonic MHC. The freeze-thaw lesion (*Wound*) occupies approximately the upper 75% of the field, while residual subendocardial myocardium (*Myo*) is present in the lower 25%. None of the grafted cells express embryonic MHC, indicating no differentiation had taken place yet. Methyl green counterstain. ×80. (*C*) 3-d graft. Multiple multinucleated myotubes (*MT*) are present. Note that myotubes are already aligned in parallel. The surrounding tissue contains numerous fibroblasts (some of which may be of graft origin), macrophages, and capillaries, characteristic of granulation tissue. Two mitotic figures are present at the lower right (*arrowheads*). Hematoxylin and eosin stain. ×800. (*D*) 3-d graft stained for embryonic MHC. The multinucleated myotubes (*MT*) express embryonic MHC, indicated by brown staining. Note faint cross-striations present at the periphery of some myotubes (*arrowheads*). Comparable staining was seen using antibodies to MHC-fast (not shown). Methyl green counterstain. ×800. (*E*) 2-wk graft. Multinucleated myofibers are present and many have peripherally placed nuclei (*arrows*); most of these nuclei appear to be within the sarcolemma, although some may be immediately external. Cross-striations were readily seen under the microscope but appear faint in the photograph. Hematoxylin and eosin staining. ×800. (*F*) 2-wk graft stained for embryonic MHC. The myofibers of the graft stain vigorously for embryonic MHC, while the underlying granulation tissue (*Wound*) and subendocardial myocardium (*Myo*) do not stain. Methyl green counterstain. ×80. (*G*) 2-wk graft stained for fast fiber isoforms of MHC. There is intense staining of the engrafted myofibers (*Graft*), indicating that they exhibit a fast twitch phenotype. Note that the residual myocardium (*Myo*) beneath the graft does not stain, nor does the granulation tissue of the injured region (*Wound*). ×80. (*H*) 7-wk graft stained for embryonic MHC. The graft continues to stain vigorously for embryonic MHC. There is no staining in the underlying young scar tissue (*Wound*) or the residual subendocardial myocardium (*Myo*). Methyl green counterstain. ×80. (*I*) 7-wk graft stained for cardiac MHC-α. The skeletal myofibers of the graft do not express MHC-α, nor does the underlying scar tissue (*Wound*). This indicates that the grafted skeletal muscle does not show cardiac differentiation. The subendocardial myocardium (*Myo*) stains vigorously for MHC-α. Methyl green counterstain. ×80. (*J*) 7-wk graft. Mature myofibers are present. Most myofibers have peripheral nuclei. Cross-striations were readily apparent under the microscope, but again are faint in the photograph. Multiple capillaries are present within the muscle tissue (*arrows*). Hematoxylin and eosin stain. ×800. (*K*) 3-mo graft. The myofibers (obliquely and cross-sectioned) have peripheral nuclei and are closely apposed with little intervening extracellular matrix. The myofibers are hypertrophic compared with the 7-wk grafts (compare fiber diameter with *J*). Most 3-mo grafts had this appearance. Hematoxylin and eosin stain. ×800. (*L*) 3-mo graft. The myofibers (cross-sectioned) in this region are encased by dense scar tissue and are atrophic. Note the markedly diminished cell diameters compared with *K*. Such entrapment of myofibers by scar was seen in one region of one heart. Hematoxylin and eosin stain. ×800.

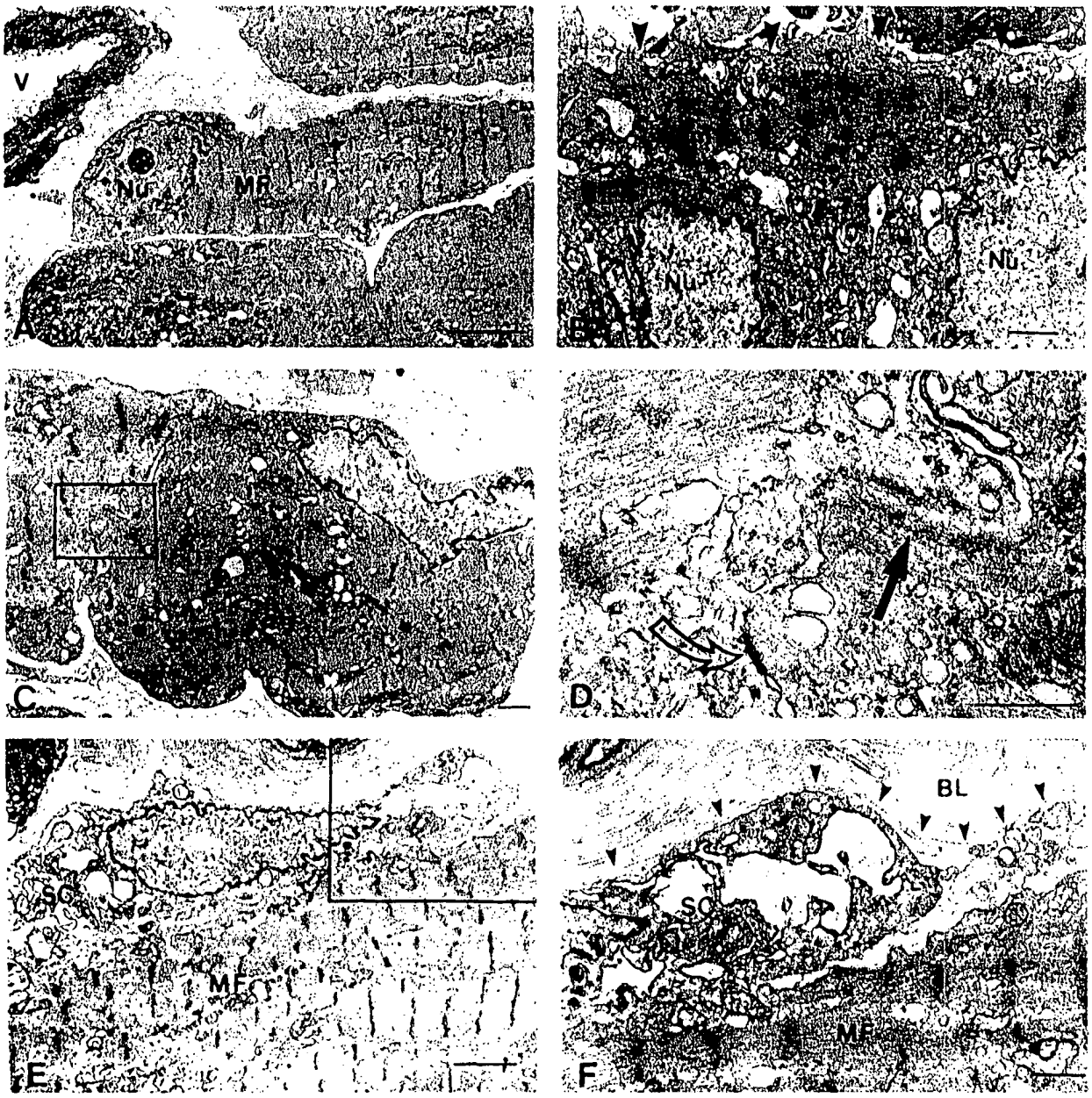

Figure 2. Transmission electron micrographs of 2-wk-old myoblast graft. The graft was placed immediately after cardiac freeze-thaw injury. (*A*) Low magnification overview showing well differentiated, striated skeletal myofibers (*MF*) within a collagen-rich matrix. A small venule (*V*) is shown at the left aspect. *Nu*, nucleus. Bar, 5 μm. (*B*) Moderately differentiated skeletal myofiber containing two nuclei (*Nu*), a modest complement of myofibrils (*mf*), and abundant ribosomes and sarcoplasmic reticulum between the nuclei. The sarcolemma is delimited by arrowheads. Bar, 1 μm. (*C*) Intercellular junction formation between adjacent myofibers. The two cells have closely apposed and interdigitated membranes. Two electron-dense plaques between the cells are present within the boxed region, suggestive of an adherens type intermediate junction and a gap junction, shown at higher magnification in *D*. Bar, 1 μm. (*D*) Higher magnification of the junctional region boxed in *C*, showing putative intermediate junction between adjacent myofibers (*solid arrow*) and gap junction (*open arrow*). Bar, 0.5 μm. (*E*) Skeletal myofiber (*MF*) with closely apposed mesenchymal cell atop it, suggestive of a satellite cell (*SC*). The boxed region is shown at higher magnification in *F*. Bar, 2 μm. (*F*) Higher magnification of region boxed in *E*. The putative satellite cell (*SC*) and the myofiber (*MF*) are contained within the same basal lamina compartment (*BL*, outlined by *arrowheads*). Although the cell has abundant rough endoplasmic reticulum, its location within the basal lamina of the myofiber and the absence of fibrillar collagen from this space make it unlikely that this is a fibroblast. Bar, 1 μm.

more rapidly when cells were transplanted into an injury with more advanced healing.

Proliferation of myoblast grafts. To identify cells undergoing DNA synthesis, the thymidine analogue BrdU was administered for 24 h before time of killing in most groups; animals in the day 1 group received a single pulse of BrdU 1 h before time of killing. Double immunostaining was performed with antibodies to the fast isoform of MHC and to BrdU, to detect

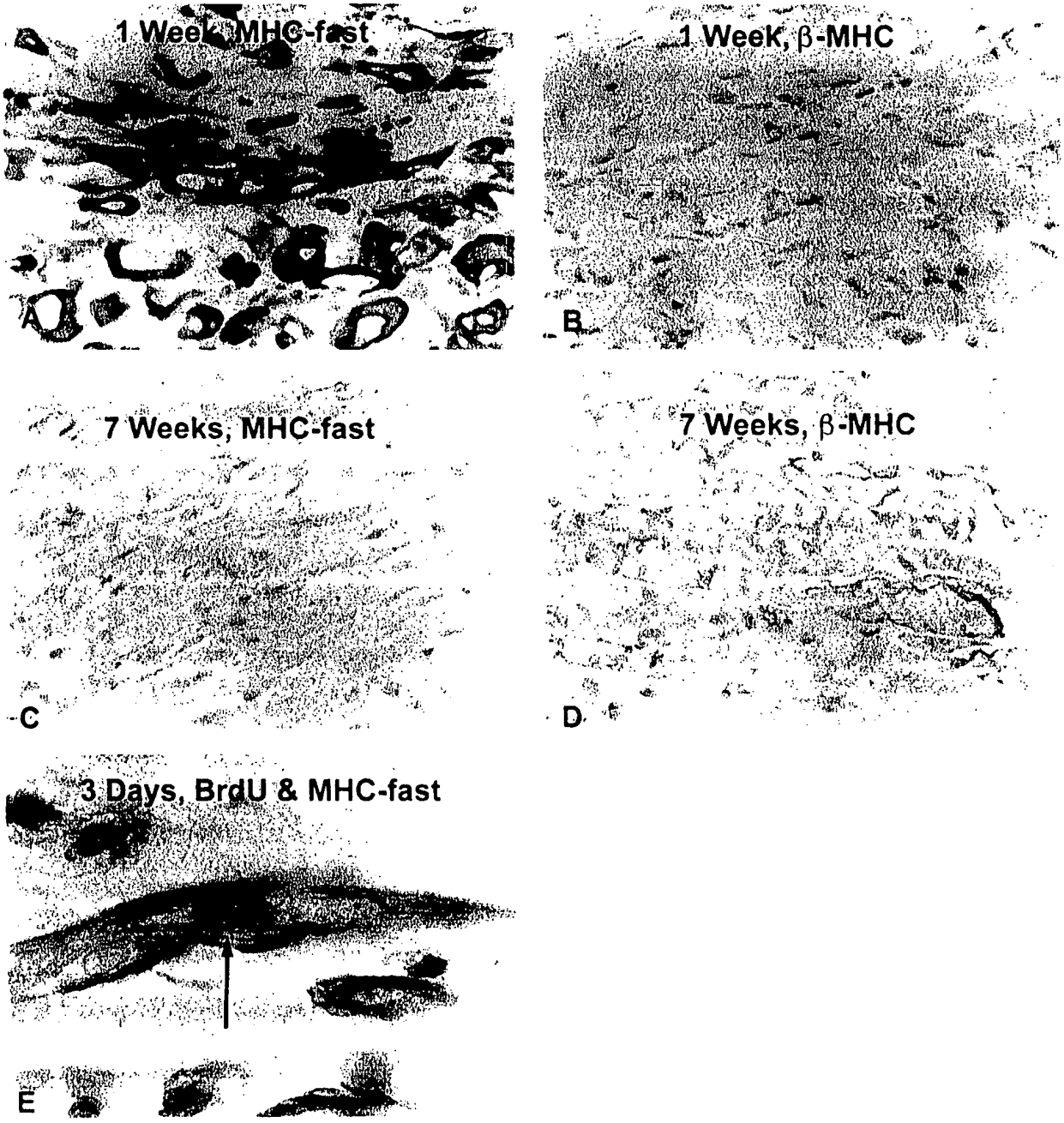

Figure 3. Fiber type conversion and proliferation of engrafted skeletal myoblasts. For the fiber typing experiments, rat hearts were injured by freeze-thaw and the lesions were allowed to heal for 1 wk. Syngeneic skeletal myoblasts were engrafted into the 1-wk-old wounds. For studies of cell proliferation, myoblasts were engrafted immediately after cardiac injury. Rats were killed at the indicated times after transplantation. Antibodies specific to fast twitch (MHC-fast) and slow twitch (β-MHC) fibers were used to define fiber types. Processing for frozen sections in *A–D* resulted in formation of contraction bands, artifactual clumping of the sarcomeres due to hypercontracture. BrdU was administered 24 h before time of killing to detect DNA synthesis. Double immunostaining for BrdU and MHC was then performed on paraffin sections. Appearance of a BrdU-positive nucleus within a myosin-positive cell indicated the myoblast had replicated and fused into the myotube within the last 24 h. (*A*) 1-wk graft stained for fast fiber isoforms of MHC. There is intense staining of the engrafted cells, indicating a fast fiber phenotype. Hematoxylin counterstain. ×960. (*B*) 1-wk graft stained with an antibody to the slow fiber-specific β-MHC. None of the grafted cells express β-MHC at this time, indicating that the cells show no characteristics of slow fibers. Methyl green counterstain. ×960. (*C*) 7-wk graft stained with an antibody to MHC-fast. There is weak staining compared with the 1-wk graft (*A*). Methyl green counterstain. ×960. (*D*) 7-wk graft stained with an antibody to β-MHC. The grafted cells now express β-MHC, indicating that they are acquiring a slow fiber phenotype (compare with *B*). Methyl green counterstain. ×960. (*E*) 3-d graft doubly stained for BrdU (purple) and MHC-fast (brown). One nucleus within the myotube stains purple (*arrow*), indicating it has undergone DNA replication before fusion into the myotube. The remaining nuclei in the myotube do not contain BrdU and pick up the red counterstain. Numerous myosin-negative cells in the surrounding wound tissue also stain positively for BrdU. Nuclear fast red counterstain. ×2,400.

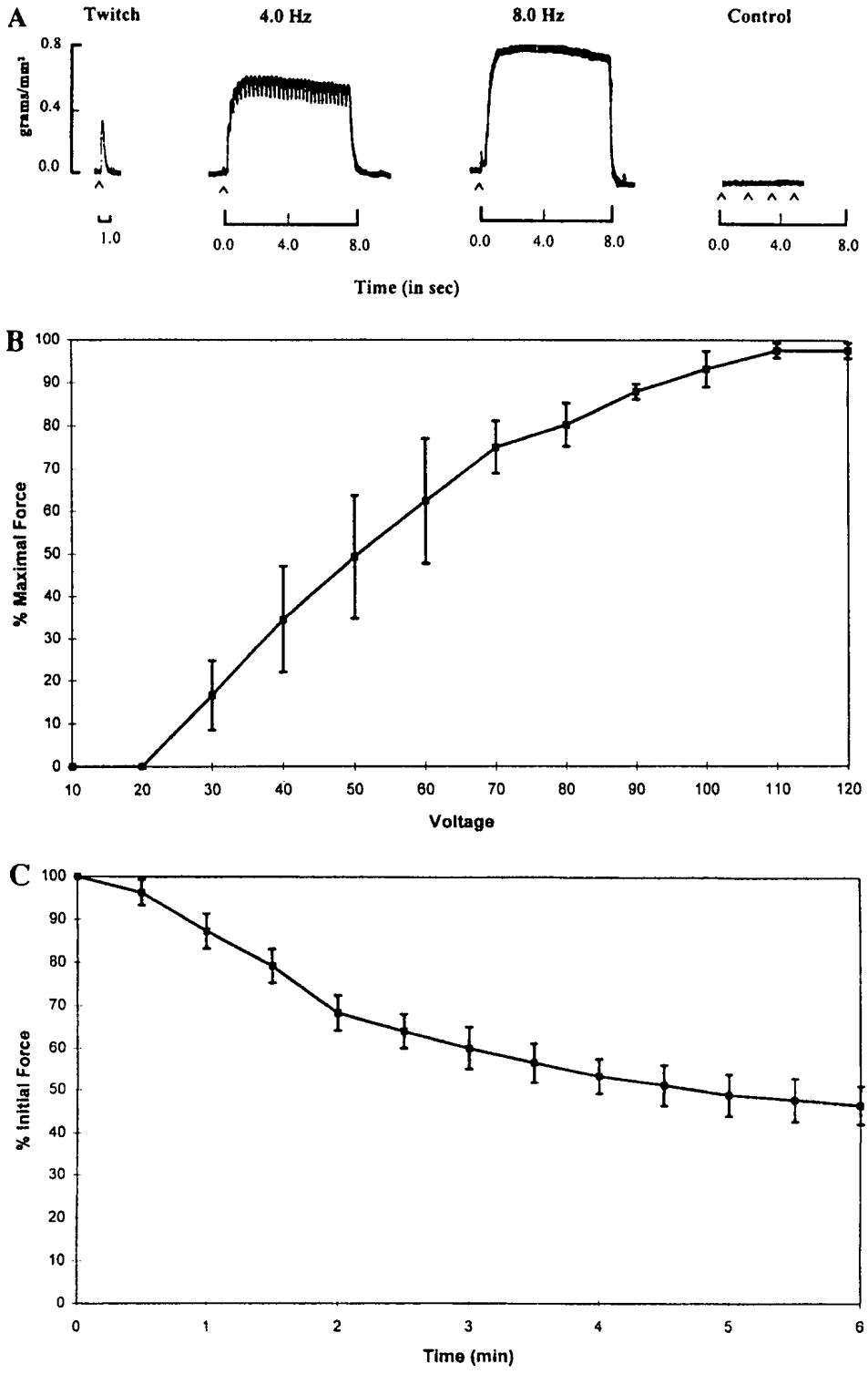

Figure 4. Contractile function of myoblast grafts ex vivo. Wound strips from injured hearts receiving either skeletal myoblasts or a sham saline injection were excised at 2 wk. Wounds were mounted on an isometric tension myograph in oxygenated buffer and electrically stimulated. The carats indicate the onset of electrical stimulation. Force has been normalized to cross-sectional area. (*A*) The first panel shows individual muscle twitch in a myoblast-injected wound. Note the rapid contraction and relaxation rates. The second panel shows that individual twitches began to superimpose with a stimulation frequency of 4 Hz, with a resulting potentiation in developed tension. The third panel shows that tetanus was induced with stimuli ≥ 7 Hz. Note the further increase in tension compared with the 4-Hz stimulation. Time to peak force in this preparation was ~ 1 s, faster than was typically observed for the overall group. The fourth panel shows that no tension was developed at any voltage in a sham-injected wound. This tracing is representative of six wound strips from three sham-injected hearts. (*B*) Force–voltage relationship. Developed tension for individual twitches increased as stimuli increased from 30 to 100 V, indicating recruitment of additional myofibers. Data have been normalized to maximal developed tension and are presented as mean±SEM of eight wound strips from six hearts. (*C*) Fatigue test. Wounds containing myoblast grafts were subjected to a cardiac-like duty cycle, consisting of repeated episodes of 0.33 s of tetanus/0.67 s of rest, to mimic a heart rate of 60 beats/min. There was a 53% decrease in developed tension at the end of the 6-min test. Note that most of the diminution in force occurred during the first 3 min. Data represent mean±SEM of seven wound strips from five hearts.

myoblasts which had proliferated and subsequently differentiated. In the day 1 grafts, proliferating cells were present within the necrotic lesion, which could have represented either graft cells or macrophages. As mentioned above, none of the cells expressed MHC at this time, so it was not possible to determine which among these were myoblasts (versus transplanted fibroblasts or host macrophages). In the day 3 grafts, occasional BrdU-positive nuclei were identified within myosin-pos-

itive cells (Fig. 3 *E*). We observed a total of 12 such nuclei in three hearts. No attempt was made to quantify this low rate, but it was certainly < 1% of total nuclei in myosin-positive cells. Virtually no BrdU-positive nuclei were seen in myosin-positive cells at 1, 2, or 7 wk after transplantation (not shown). We conclude that myoblast proliferation occurs for at least 3 d after grafting, but by 1 wk virtually all cells have ceased replicating.

Contractile function of myoblast grafts. The contractile properties of 2-wk-old myoblast grafts were determined by attaching isolated wound strips to a tension myograph ex vivo. Virtually no spontaneous mechanical activity was detected, consistent with the paucity of cardiomyocytes histologically. Electrical stimulation caused muscle twitches in six of eight myoblast-engrafted hearts (Fig. 4 *A*, *first panel*); strips from the remaining two hearts may have been damaged during sample preparation, since skeletal muscle was present histologically. The grafts showed a stepwise increase in tension development as voltage was increased from 30 to ~ 100 V with a plateau thereafter (Fig. 4 *B*). This indicates that increasing voltage recruited additional myofibers to contract, implying that the graft myofibers are electrically insulated from one another. It should be noted that cardiac muscle does not increase contractile force with increasing voltage, since cardiocytes are coupled electrically via gap junctions.

Next, force-frequency relationships were determined. Using 120% of the voltage required for maximal tension development, the frequency of stimulation was increased incrementally from 0.5 to 10 Hz. Twitches began to superimpose at frequencies of 3–4 Hz, with a resulting increase in total developed tension (Fig. 4 *A*, *second panel*). Fully fused tetani were produced with 6–7 Hz stimulation (Fig. 4 *A*, *third panel*). Peak force during tetanus was 1.98±0.45 grams (mean SEM); after normalization to cross-sectional area the peak force was 0.72±0.14 grams/mm^2. The time to peak tetanic force averaged 2.3±0.3 s, although 90% of peak force was typically generated within 1.5 s. The time to half-relaxation after tetanus was 240±17 ms. It should be stressed that tetanus cannot be induced in cardiac muscle, due to the long refractory period of cardiocytes.

Finally, a fatigue test was performed to test the response of this muscle to a cardiac-like work load. The grafts were subjected to a duty cycle consisting of repeated 0.33 s of tetanic stimuli followed by 0.67 s of rest, mimicking a heart rate of 60 beats/min. The grafts showed a 32% decline in developed tension by 2 min and a 53% decline by the end of the 6-min test period (Fig. 4 *C*). No contractile activity could be elicited from six of seven wound strips from three injured hearts which received a sham injection of saline instead of myoblasts (Fig. 4 *A*, *fourth panel*). In one sham heart an adhesion had developed between the heart and chest wall, resulting in a small amount of intercostal muscle adhering to one of the two wound strips. In this preparation we detected a peak force of 0.04 grams/mm^2, < 2% of what was present in the myoblast-engrafted hearts.

Thus, the skeletal muscle grafts could be stimulated to contract ex vivo and could sustain a cardiac-like duty cycle over a 6-min test period. Furthermore, the grafts showed two physiological properties unique to skeletal muscle: recruitment of fibers with increasing voltage and the ability to sustain tetanic contraction. We do not know yet whether the grafts contract in vivo.

Discussion

The principal findings of this study are: (*a*) neonatal skeletal myoblasts can be grafted into an injured heart; (*b*) the engrafted myoblasts initially proliferate and then begin to form multinucleated myotubes by day 3; (*c*) the myotubes differentiate into mature myofibers, which initially have a phenotype similar to fast twitch muscle; (*d*) the myofibers develop characteristics of slow twitch muscle as the wound heals; (*e*) the new muscle may form satellite stem cells; and (*f*) the new muscle can be stimulated to contract ex vivo.

Strategies for muscle regeneration after myocardial injury. In principal, there are at least three strategies to induce muscle regeneration after myocardial infarction. First, the surrounding cardiac myocytes could be stimulated to migrate into the wound and proliferate to repair the defect. There is evidence that a limited amount of cell replication by adult cardiocytes occurs naturally after myocardial infarction in humans (17) and in rats (18, 19), but the response is clearly not adequate to repair the defect. The factors responsible for cell cycle arrest in cardiocytes are not well enough defined at present to begin exploring this as a therapy. (The interested reader is referred to references 20–23 for further information on this topic.)

A second strategy is to induce the cells of cardiac granulation tissue (the fibroblast-rich tissue of wound repair) to differentiate into muscle rather than forming a scar. There is not enough known about cardiac differentiation at present to attempt formation of new myocardium. However, much more is known about skeletal muscle determination. The discovery of myogenic determination genes (24, 25) has made it possible to induce a wide range of cultured cell types to differentiate into skeletal muscle. Recent studies from our group (26) and others (27) have shown that cells in cardiac granulation tissue can be induced to differentiate into skeletal muscle by transfection with the prototype myogenic determination gene, MyoD. In these early experiments, however, the frequency of muscle differentiation has been low after MyoD gene transfer. Until the frequency of myogenic conversion can be increased, it will be difficult to investigate the functional properties of the MyoD-induced skeletal muscle.

The third strategy for muscular repair of infarcts is to transplant either skeletal or cardiac myoblasts into the injured region. Studies by Koh et al. (3) and Soonpaa et al. (2) have demonstrated that fetal cardiocytes will form intercalated discs with host cardiocytes, including gap junctions and adherens junctions, when transplanted into normal hearts. No proliferation was detected in the grafted cardiocytes. Less information is available on grafting of cardiocytes into injured hearts. Our group (28) and others (29, 30) have preliminary data showing that neonatal rat or fetal human cardiocytes can be transplanted successfully into injured rat hearts. To our knowledge there is no information regarding proliferation of these grafts, nor are any functional data available. As discussed above, the principal limitation to this approach is the inability to induce cardiocytes to proliferate in culture. Until cardiocytes can replicate in vitro, or proliferation-competent cells can be induced reliably to differentiate into cardiocytes, cardiocyte grafting will not be feasible in humans.

In contrast to cardiocytes, proliferating skeletal muscle precursors are readily available, either as primary myoblasts in developing muscle or as satellite cells from quiescent muscle. In this study six rat pups yielded the myoblasts implanted into

27 injured hearts. In addition to their growth in culture, the myoblasts proliferated in vivo for several days after transplantation (Fig. 3 *E*). These properties have led us and several other groups to explore skeletal muscle grafting for cardiac repair. Koh et al. (7) demonstrated that the myogenic cell line C2C12 could be transplanted into the hearts of normal syngeneic mice, where the cells fused to form multinucleated myofibers. The same group also demonstrated that C2C12 cells stably transfected with a plasmid encoding active TGF-β could induce angiogenesis around the graft site (31). No coupling between the host cardiocytes and the grafted skeletal muscle was observed in either experiment.

Chiu et al. (8, 9) transplanted autologous satellite cells into cardiac freeze-thaw lesions in dogs. Comparable with our study, they also found that the grafts formed muscle cells within the healing lesion. In distinction to the current study, however, they hypothesized that their grafted skeletal muscle cells differentiated into cardiac muscle, via "milieu-dependent effects." The evidence for a cardiac phenotype was that some cells within the grafts had central rather than peripheral nuclei, and some cells contained refractile transverse structures under light microscopy interpreted to be intercalated discs. Although we observed some myofibers with persistent central nuclei in this study, as well as rare cells showing intermediate and gap junctions (Fig. 2, *C* and *D*), no intercalated discs were present by electron microscopy. More importantly, the grafted cells expressed skeletal muscle–specific proteins and failed to express the cardiac-specific isoform MHC-α up to 3 mo after transplantation. Thus, there clearly was no cardiac differentiation in this study.

Conversion of grafts from fast to slow twitch muscle. Although the skeletal muscle grafts expressed the fast fiber isoform of MHC at 1 and 2 wk, they expressed β-MHC, a marker for slow twitch fibers, at 7 wk and 3 mo. This indicates that the grafts were converting to slow twitch fibers. Conversion was apparently more rapid when the myoblasts were injected into wounds where healing had been allowed to progress for 1 wk, as opposed to immediately after injury. In the delayed transplantation model the grafts expressed β-MHC at 2 wk, while in the immediate transplantation model this protein was not detected until 7 wk. It is possible that the growth factors and cytokines present in the early wound delay myoblast differentiation and subsequent fiber type conversion.

Slow fibers exhibit several important differences from fast fibers, including a slower shortening velocity, use of oxidative phosphorylation for ATP production, a higher mitochondrial content, a higher myoglobin content, and a much greater resistance to fatigue (16, 32). An interesting parallel is that the latissimus dorsi muscle also undergoes fiber type switching when it is conditioned for dynamic cardiomyoplasty. Cardiomyoplasty is an experimental therapy for heart failure, where skeletal muscle is wrapped around the heart to serve as a ventricular assist device (33). Untrained latissimus dorsi is a mixed fiber type muscle which fatigues rapidly with repeated stimulation. When conditioned by repeated electrical stimulation for 6 wk before surgery, however, the latissimus dorsi converts entirely to slow twitch fibers and becomes fatigue resistant (6). Only the conditioned, slow twitch muscle is able to assist cardiac function. This parallel suggests the intriguing possibility that repeated electromechanical stimulation leads to activation of the slow fiber phenotype. Since we did not test whether the environment of the heart contributed to fiber type conversion, additional experiments will be required to determine the mechanism. The fact that the grafts differentiated into slow twitch fibers suggests that they may be suited to perform a cardiac type work load.

Will skeletal muscle transplantation augment cardiac function? This study definitively showed that myoblast grafting can generate new contractile tissue. The skeletal muscle grafts exhibited characteristic twitches when stimulated ex vivo (Fig. 4 *A*) and showed recruitment of contractile units with increasing voltage (Fig. 4 *B*). Furthermore, tetanus could be induced with rapid stimulation (Fig. 4 *A*, *second* and *third panels*), and the grafts could perform a cardiac-like duty cycle for 6 min (Fig. 4 *C*). Peak force during tetanus averaged 0.7±0.1 grams/mm^2. Since the wound strips contained < 50% of the myofiber content of normal muscle, due to inclusion of scar tissue, the force can be normalized to at least 1.4 grams/mm^2 muscle. Adult mammalian muscle can generate 15–35 grams/mm^2 force at tetanus, depending on fiber type (14, 34). Thus, the 2-wk grafts generated ~ 4–10% of the predicted force for mature skeletal muscle. Several factors may cause a lower than predicted force, including the relative immaturity of the 2-wk myofibers, stretching of the immature extracellular matrix, poor cell matrix attachments, or misalignment of some fibers relative to the axis of the wound strip.

Although preliminary, these results are encouraging and suggest that more detailed studies of contractile function are warranted in skeletal myoblast-engrafted hearts. A critical question is whether the skeletal muscle grafts contract in vivo. To provide coordinated mechanical assistance, the grafted cells ideally should form electrical and mechanical junctions with the host myocardium. In our grafts the skeletal muscle cells were insulated from the remaining myocardium by scar tissue, so there was no opportunity for myofiber–cardiocyte coupling to occur. Koh et al. (7) transplanted C2C12 myoblasts into normal mouse hearts and observed no cell junctions between grafted myofibers and host cardiocytes by electron microscopy. Although proliferating myoblasts have been reported to synthesize both gap junction proteins (35) and *N*-cadherin (36, 37), these proteins are typically absent from adult skeletal myofibers. By electron microscopy we observed evidence both for intermediate and gap junction formation between skeletal myofibers 2 wk after grafting (Fig. 2, *C* and *D*). This finding was infrequent, however, and it is unknown whether such junctions would persist in longer term grafts. If skeletal muscle will not couple spontaneously with cardiac muscle, it is possible that such junctions could be induced by stably transfecting skeletal muscle cells with genes for cardiac junctional proteins. Another possibility is that skeletal muscle grafts could be electrically paced in synchrony with the cardiac cycle. Pacing would require sufficient voltage to activate all of the fibers, and currently it is unknown whether this would have a deleterious effect on the surrounding myocardium.

In the uninjured heart there is a complex fiber geometry, where the outer fibers run in the long axis, the midwall fibers run in the short axis, and the inner fibers again run in the long axis. This geometry is established during embryogenesis and is thought to be important for mechanical efficiency. In this study, the grafted myofibers were predominantly aligned with the short (transverse) axis of the heart. Alignment was noted as early as day 3, when myotube formation was prominent (Fig. 1 *C*). This is the same orientation that wound fibroblasts and collagen fibers acquire during wound healing, and it seems

likely that all are aligned by local mechanical forces. It is not known whether alignment with the heart's short axis will influence the ability of these myofibers to restore mechanical function after injury.

There are two aspects of skeletal muscle which theoretically could make it superior to cardiac muscle for infarct repair. First, skeletal muscle is much more resistant to ischemia than cardiac muscle. Skeletal muscle can withstand many hours of severe ischemia without becoming irreversibly injured, whereas in myocardium irreversible injury begins within 20 min (38). A second difference is that skeletal myoblast grafts might establish satellite cells. Satellite cells are the resident stem cells in skeletal muscle and proliferate in response to injury. Once activated, satellite cells can fuse with damaged myofibers or establish new myofibers to replace those lost to necrosis. We observed cells within 2-wk grafts which were morphologically consistent with satellite cells by electron microscopy (Fig. 3, *E* and *F*). Thus, it is possible that infarcts repaired with skeletal myoblasts might become more resistant to a subsequent episode of ischemia or might be able to replace myofibers damaged by ischemia.

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Regeneration of Human Infarcted Heart Muscle by Intracoronary Autologous Bone Marrow Cell Transplantation in Chronic Coronary Artery Disease

The IACT Study

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OBJECTIVES Stem cell therapy may be useful in chronic myocardial infarction (MI); this is conceivable, but not yet demonstrated in humans.

BACKGROUND After acute MI, bone marrow-derived cells improve cardiac function.

METHODS We treated 18 consecutive patients with chronic MI (5 months to 8.5 years old) by the intracoronary transplantation of autologous bone marrow mononuclear cells and compared them with a representative control group without cell therapy.

RESULTS After three months, in the transplantation group, infarct size was reduced by 30% and global left ventricular ejection fraction (+15%) and infarction wall movement velocity (+57%) increased significantly, whereas in the control group no significant changes were observed in infarct size, left ventricular ejection fraction, or wall movement velocity of infarcted area. Percutaneous transluminal coronary angioplasty alone had no effect on left ventricular function. After bone marrow cell transplantation, there was an improvement of maximum oxygen uptake (Vo_{2max}, +11%) and of regional ^{18}F-fluor-desoxy-glucose uptake into infarct tissue (+15%).

CONCLUSIONS These results demonstrate that functional and metabolic regeneration of infarcted and chronically avital tissue can be realized in humans by bone marrow mononuclear cell transplantation. (J Am Coll Cardiol 2005;46:1651–8) © 2005 by the American College of Cardiology Foundation

Cardiac performance after myocardial infarction (MI) is compromised by ventricular remodeling, which represents a major cause of late infarct-related chronic heart failure and death (1,2). Although conventional drug therapy (e.g., with beta-receptor blockers and/or angiotensin-converting enzyme inhibitors) may delay remodeling, there is no basic

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therapeutic regimen available for preventing or even reversing this process. By the use of interventional therapeutics (percutaneous transluminal coronary angioplasty [PTCA], stent), recanalization of the occluded infarct-related artery is possible, thereby improving or normalizing coronary blood flow. However, despite sufficient reperfusion of infarcted tissue, the viability of the infarcted myocardium cannot, or can only insufficiently, be improved in most of these patients (3). Therefore, catheter-based therapy of acute MI is useful for vascular recanalization, but the second and crucial step, the regeneration of necrotic heart muscle, is not realized by this vascular procedure alone.

Experimental (4) and clinical (5,6) studies have shown recently for the first time that bone marrow mononuclear cells (BMCs) may regenerate damaged myocardium in acute MI in humans. Because the regenerative potential of bone marrow-derived cells ought also to be expected to exist in chronically ischemic heart disease as well (7–12), we have assembled in an ongoing clinical investigation 18 patients with chronic MI to prove this new therapeutic possibility.

METHODS

Study population. All 18 patients (49 ± 11 years) were men and were recruited consecutively from January 2003 until March 2004. They had had transmural MI 27 ± 31 months before, at which point all infarcts had been treated acutely by PTCA and/or stent implantation (Table 1, Fig. 1).

The inclusion criteria were age <70 years, one-vessel disease with an open infarct-related artery at the time of stem cell therapy, sinus rhythm, a clear-cut demarcation of the ventriculographic infarct area, and no coronary bypass surgery. General exclusion criteria were severe comorbidity and alcohol or drug dependency. Although chronically infarcted myocardium usually does not regenerate sponta-

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Abbreviations and Acronyms

BMC = bone marrow mononuclear cell
CPK = creatine phosphokinase
ECG = electrocardiogram
LV = left ventricular
MI = myocardial infarction
PET = positron emission tomography
PTCA = percutaneous transluminal coronary angioplasty
Tx group = transplantation group

neously, for comparison a control group, parallel to the recruitment of the stem cell transplantation group (Tx group), was recruited and analyzed, meeting the same inclusion criteria as the stem-cell group. The recruitment of patients was performed according to a randomization procedure in which all patients of the entire chronic infarction group were distributed to the treatment group, where they agreed with all the therapeutic regimen. Alternatively, all patients of the chronic infarction group who refused the therapeutic regimen (bone marrow puncture and aspiration, intracoronary cell transplantation, and another cardiac catheterization) were allocated to the control group. All medications with angiotensin-converting enzyme inhibitors and with beta receptor blockers were maintained constant during the study period.

The cell-treated patients had stable ventricular dynamics for infarct size, ejection fraction, and wall movement velocity of infarcted area at least 9 ± 6 months before cell transplantation. Infarct size at the time of cell therapy showed an amount of 27 ± 8% of the circumference of the left ventricle (LV), determined by ventriculography.

Preparation of BMCs. One day before cell therapy, bone marrow was taken (80 ml from the iliac crest) and mononuclear cells were isolated and identified including CD34-positive cells, AC133-positive cells and CD45/CD14 negative cells (6). The cells were isolated under good manufacturing practice conditions by Ficoll density separation on Lymphocyte Separation Medium (Bio Whittaker, Walkersville, Maryland), before the residual erythrocytes were lysed with H_2O. For overnight cultivation, 1×10^6 BMCs/ml were placed in Teflon bags (Vuelife, Cell Genix, Gaithersburg, Maryland) and cultivated in X-Vivo 15 Medium (Bio Whittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed three times with heparinized saline before final resuspension in heparinized saline. Viability was 93 ± 3%. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. These cells were used for therapy. All microbiologic tests of the clinically used cell preparations proved negative. All patients received extensive information about the procedure, which was approved by the ethical committee of our university, and all gave written informed consent.

Administration of BMCs. Following assessment of baseline examinations (coronary angiography, left ventriculography, spiroergometry, ^{99m}Tc-tetrofosmin single-photon emission computed tomography (SPECT) and ^{18}F-fluor-deoxy-glucose (^{18}F-FDG) positron emission tomography (PET), cell transplantation was performed via the intracoronary administration route (6,13) using four to six fractional infusions parallel to balloon inflation over 2 to 4 min of 3 to 5 ml of cell suspension, each containing 15 to 22 × 10^6 mononuclear cells. All cells were infused directly into the infarcted zone through the infarct-related artery via an angioplasty balloon catheter, which was inflated at a low pressure (2 to 4 atm) and was located within

Table 1. Demographic Data of Intracoronary Bone Marrow Stem Cell Transplantation Group and Control Group

Characteristics	Tx Group	Control Group	p
No. of patients	18	18	
Age, yrs	49 ± 11	52 ± 10	NS
Transmural myocardial infarction, months before Tx	27 ± 31	30 ± 34	NS
Coronary angiography			
LAD/LCX/RCA as affected vessel	16/0/2	10/3/5	
No. of patients with stent implantation	16	17	NS
Risk factors			
Diabetes mellitus, %	16	11	NS
Positive family history, %	44	33	NS
Smoker and ex-smoker, %	67	56	NS
Hyperlipoproteinemia, %	89	94	NS
Medication			
Beta-blocker, %	94	89	NS
Angiotensin-converting enzyme inhibitor, %	94	89	NS
Statin, %	94	100	NS
Laboratory parameters			
CPK, U/l	1,504 ± 979	1,489 ± 952	NS
Bone marrow mononuclear cells, n (10^6×)	90		

Values are mean ± SD or number of patients.
CPK = creatine phosphokinase; LAD = left anterior descending coronary artery; LCX = left circumflex coronary artery; RCA = right coronary artery; Tx = intracoronary bone marrow stem cell transplantation.

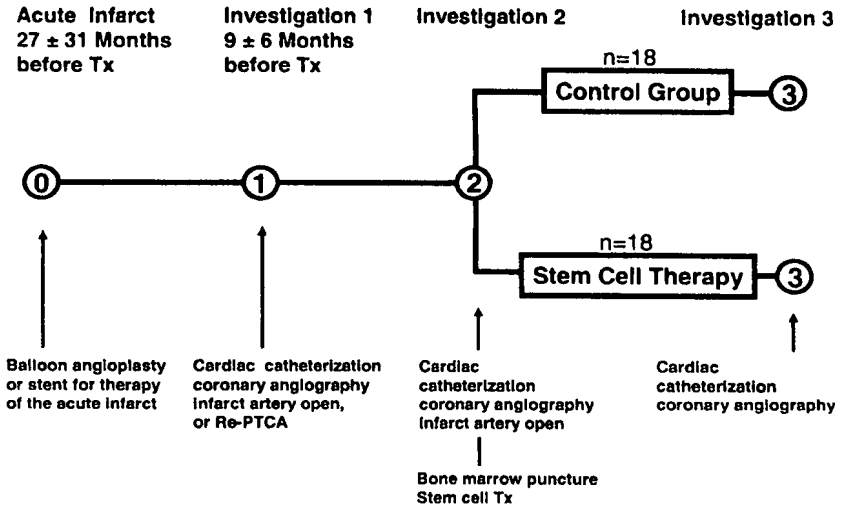

Figure 1. Diagrammatic representation of the algorithm of intracoronary stem cell therapy (Tx) in chronic ischemic heart disease after myocardial infarction. The infarcts occurred 27 ± 31 months before Tx. All infarct patients were treated with percutaneous transluminal coronary angioplasty (PTCA) or with stent implantation. 9 ± 6 months before (investigation 1) coronary angiography (including quantitative left ventriuclography) was performed. If re-stenosis was present, re-PTCA was made. Investigation 2 embraces all patients for the evaluation of coronary morphology after PTCA/stent. Only patients with an open infarct-related artery were included in both groups. Patients who agreed to Tx received within 10 days after investigation 2 bone marrow punctures and Tx by the intracoronary administration route and had altogether five invasive investigations, including two for therapeutic reasons (nos. 0 and 1). Patients who were not eligible for Tx (disagreement with bone marrow puncture and with subsequent Tx) served as a control group. Investigation 3 represents all follow-up measurements 3 months after Tx (Tx patients) or after investigation 2 for control group patients.

the previously stented coronary segments. This prevented backflow of cells and produced stop flow beyond the site of balloon inflation to facilitate high-pressure infiltration of cells into the infarcted zone. Prolonged contact time for cellular migration was also enabled. Three months after catheter-guided cell transplantation, all functional tests were repeated, including coronary angiography and left ventriculography. There were no procedural or cell-induced complications, and there were no side effects in any patient.

Spiroergometry. Aerobic exercise capacity was examined before (<10 days) intracoronary cell transplantation and three months later during follow-up. All patients (n = 18) were subjected to initial bicycle spiroergometry to assess their functional fitness and to determine the limit of safe intensity of exercise. We chose a protocol with an intensified workload up to the symptom-limited maximum (basic load of 50 W, intensification at 25 W, 2-min duration of each workload step). We determined the anaerobic threshold for prescribing a suitable load intensity. During the whole spiroergometry, monitoring by a 12-lead electrocardiogram (ECG) was carried out. The exercise capacity was assessed on the basis of maximum load levels expressed in watts (W_{max}) and maximum peak oxygen uptake (Vo_{2max}).

Coronary angiography and left ventriculography. Coronary angiography and biplane left ventriculography were performed 9 ± 6 months before cell transplantation and also a second time, within 10 days, immediately before cell therapy. The therapeutic follow-up was three months after the treatment. Thus, stable baseline conditions were documented (coronary vessel involvement, ventricular function, and geometry). Cardiac function was evaluated by left ventricular (LV) ejection fraction and by auxotonic myocardial contractility index, evaluated by the wall movement velocity of the infarcted area. The infarct size was calculated according to the method of Sheehan (14) by plotting five axes perpendicular to the long axis of the heart in the main akinetic or dyskinetic segment of the ventricular wall. Systolic and diastolic lengths were then measured by two independent observers, and the mean difference was divided by the systolic duration in seconds.

Quantification of coronary stenosis (restenosis). Cinecoronarangiograms were obtained during stem cell transplantation and at three months thereafter according to standard acquisition guidelines. The angiograms were evaluated by two independent observers and quantitative analysis was performed (15). Standard morphologic criteria were used to characterize the complexity of baseline lesions. The user-defined reference diameter proximal to the stenosis and the minimal luminal diameter within the culprit of the stenosis were used to calculate the percentage of stenosis. A value of 0 mm was assigned for the minimal luminal diameter in case of total occlusion at baseline or follow-up. Restenosis was defined as ≥50% stenosis of the initial target lesion at follow-up. Calculations of restenosis were performed in both groups, with and without stem cell therapy, in the same way, thus enabling evaluation the differential effects of PTCA-guided cell therapy and of PTCA effects alone.

Ventricular function after PTCA in the control group. For the evaluation of a potential effect on the PTCA intervention itself on LV function, all patients in the control group were analyzed with regard to infarct size, ejection fraction, and infarction wall movement velocity.

Table 2. Single Values of Intracoronary Bone Marrow Stem Cell Transplantation Group

Patient Number	Area of Infarction, %*			LV Ejection Fraction, %*			Infarction Wall Movement Velocity, cm/s*		
	Investigation 1 9 ± 6 Mo Before Tx	Investigation 2 <10 Days Before Tx	Investigation 3 3 Mo After Tx	Investigation 1 9 ± 6 Mo Before Tx	Investigation 2 <10 Days Before Tx	Investigation 3 3 Mo After Tx	Investigation 1 9 ± 6 Mo Before Tx	Investigation 2 <10 Days Before Tx	Investigation 3 3 Mo After Tx
1	26	26	22	56	55	60	0.88	0.77	0.82
2	28	29	26	45	43	49	2.06	1.88	2.13
3	16	16	5	64	65	71	1.45	1.50	2.10
4	27	25	14	48	50	65	1.20	1.25	2.88
5	16	14	11	66	69	71	2.25	2.77	3.75
6	16	13	6	64	66	73	1.50	1.77	2.55
7	15	18	11	57	55	63	2.78	2.65	3.13
8	28	28	20	43	44	49	3.15	3.25	4.25
9	27	27	11	46	46	64	1.61	1.65	3.30
10	20	17	14	56	58	62	2.21	2.45	3.13
11	28	25	17	42	38	52	1.91	1.88	3.00
12	33	28	21	44	47	54	2.28	2.62	3.50
13	39	37	27	50	51	59	1.25	2.50	4.90
14	29	33	27	62	62	61	1.20	1.33	2.70
15	37	37	31	48	43	53	1.83	1.56	2.50
16	29	29	24	53	54	58	1.25	1.06	3.06
17		41	35		48	55		1.66	3.00
18		35	25		45	53		0.94	1.94
Mean	26	27	19	53	52	60	1.80	1.86	2.92
SD	7	8	9	8	9	7	0.63	0.70	0.91

*Calculated from left ventriculography.
LV = left ventricular; Mo = Months; other abbreviations as in Table 1.

Nuclear cardiologic investigations (PET and SPECT). ^{18}F-FDG-positron emission tomography (^{18}F-FDG PET) was performed with a Scanditronix SCX 4096 WB-Scanner (FWHM = 6 mm transaxial, axial field of view = 4.6 cm). Patients received an oral glucose load of 1 g/kg body weight 80 ± 30 min before the intravenous application of ^{18}F-FDG (380 ± 60 MBq). The ^{18}F-FDG was administered at the time of decrease of blood glucose level <130 mg/dl. An initial transmission scan was obtained using a ^{68}Ga-filled pin source to correct the subsequent emission scans for attenuation. The data acquisition was started 45 min after administration of FDG. Image data were recorded with a 256 × 256 matrix in 3 consecutive bed positions over 15 min per position. The data were reconstructed back-projected with a Hanning filter (5 mm).

^{99m}Tc-tetrofosmin SPECT. Sixty minutes after intravenous injection of 600 ± 140 MBq of the perfusion-marker ^{99m}Tc-tetrofosmin under a "rest" condition, the images were obtained using a SPECT scanner with double-head detector (PRISM 2000, Marconi/Phillips), a low-energy, high-resolution collimator, and a 128 × 128 matrix. Image data were collected over 360° at 3° every 30 s. The images were reconstructed backprojected with a low-pass filter (order 12, cutoff 0.2).

PET and SPECT evaluation. Normalized values for FDG uptake and perfusion were calculated by comparing regional with maximum tracer uptake on the reconstructed images. We performed a regional analysis of glucose metabolism and perfusion using a set of standardized, individually adjusted circular regions of interest (diameter 18.06 mm, surface 256 mm^2). The reconstructed metabolic and perfusion images were realigned for each patient (MPI-Tool, version 3.0; Advanced Tomo Vision, Erftstadt, Germany) and were resliced according to cardiac axis (short-axis and horizontal and vertical long-axis views). The regions were positioned immediately neighboring, with no overlap, according to an overlay of the co-registrated metabolic and perfusion images. The regions covered the infarct lesion as well as normal myocardium. In this way, we generated templates of regions for each patient, which could be used for the evaluation of metabolism and perfusion, before and after BMC transplantation without further modification. According to Segall et al. (16), regions with a normalized FDG uptake <50% were rated as transmural scar and regions with an uptake of 50% to 60% as non-transmural scar.

Further analysis was restricted to regions with FDG uptake <60% in the PET scans, pursuant to our intention to focus on the effects of BMC transplantation on scar tissue.

Safety parameters. To assess any inflammatory response and myocardial reaction after cell therapy, white blood cell count, the serum levels of C-reactive protein (CRP) and of creatine phosphokinase (CPK) were determined immediately before as well as after treatment. Additional analysis was done directly after transplantation and three months later: ECG at rest, 24-h Holter ECG, and echocardiography.

Statistical analysis. All data are presented as mean ± SD. Statistical significance was accepted when $p < 0.05$. Intra-individual comparison of variables of investigation 1 (9 ± 6 months before cell transplantation for Tx group, 9 ± 5 months before investigation 2 for control patients) and investigation 2 (<10 days before cell transplantation for Tx group, no transplantation for control patients) and of variables of investigation 2 and follow-up investigation 3 (3 months after cell therapy for Tx group, 8 ± 5 months after investigation 2 for control patients) was performed using Wilcoxon rank-sum test. The missing values (Table 2) were omitted and not calculated for statistical analysis. The p values (by analysis of variance) have been given for LV ejection fraction, area of infarction, and infarction wall movement velocity. Statistical analysis was performed with SPSS-Windows 10.1 software.

RESULTS

Three months after intracoronary cell therapy, the infarct size was reduced by 30%, whereas the global LV ejection fraction increased by 15% and regional infarct wall movement velocity by 57% (Tables 2 and 3). In parallel, the clinical performance improved (Table 4), as evidenced by a higher work load demonstrated by a 11% increase in maximum oxygen uptake (Vo_{2max}). SPECT investigation presented enhanced tetrofosmin uptake in the infarcted zone by 5%, and PET examination showed enhanced glucose uptake in the infarcted zone by 15%, demonstrating regeneration of formerly avital, chronically infarcted heart muscle (Fig. 2). An unchanged or even impaired LV function was not observed in any patient.

In the control group (18 patients with chronic MI, but without stem cell therapy) no significant changes were observed in infarct size, LV ejection fraction, or wall

Table 3. Cardiac Parameters in the Transplantation Group and in Control Group at the Three Investigation Time Points

	Area of Infarction, %			LV Ejection Fraction, %			Infarction Wall Movement Velocity, cm/s		
	Control Group	Tx Group	p Value*	Control Group	Tx Group	p Value*	Control Group	Tx Group	p Value*
Investigation 1	25 ± 9	26 ± 7	0.99	53 ± 10	53 ± 8	0.87	1.95 ± 0.66	1.80 ± 0.63	0.57
Investigation 2	27 ± 9	27 ± 8	0.83	51 ± 10	52 ± 9	1.00	1.88 ± 0.76	1.86 ± 0.70	0.94
Investigation 3	26 ± 9	19 ± 9	0.02	52 ± 10	60 ± 7	0.02	1.91 ± 0.79	2.92 ± 0.91	0.001

*Analysis of variance.
Abbreviations as in Table 1.

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Table 4. Positron Emission Tomography and Spiroergometry Before and After Stem Cell Therapy in Chronically Infarcted Myocardium

	^{18}F-FDG-Positron Emission Tomography		VO_{2max}, Spiroergometry	
	FDG Uptake, %	Difference in %	ml/min	Difference in %
Investigation 1	none		none	
Investigation 2	43.8 ± 8.0	+ 15	1,602 ± 533	+ 11
Investigation 3	50.5 ± 11.6		1,776 ± 523	
p (Wilcoxon test)	0.012		0.0001	

^{18}F-FDG = ^{18}F-fluor-deoxy-glucose; VO_{2max} = maximum oxygen uptake.

movement velocity of the infarcted area (Figs. 3A to 3C). Electrocardiogram at rest and on exercise and 24-h Holter ECG revealed no rhythm disturbances at any time point. Only 1 patient (from 18 cell-treated patients, 6%) developed relevant restenosis due to quantitative angiographic criteria. The restenosis could be treated adequately by stent implantation. The other 17 patients showed good patency rates without restenosis after PCI and cell transplantation. They also revealed no alterations in LV function 8 ± 5 months after PTCA.

There was no inflammatory response or myocardial reaction (white blood cell count, CRP, CPK) after cell therapy, despite a moderate increase in CRP (before cell transplantation 0.58 ± 0.48 mg/dl, after cell transplantation 1.07 ± 0.73 U/l, p = 0.002), which is usual after bone marrow puncture and/or cardiac catheterization.

DISCUSSION

The results of these investigations demonstrate, for the first time, that the intracoronary transplantation of autologous bone marrow mononuclear cells may reduce infarct size and improve LV function as well as myocardial glucose uptake in chronic ischemic heart disease attributable to chronic MI (5 months to 8.5 years old). Infarct size decreased in all patients and cardiac performance (ejection fraction, wall movement velocity of infarcted area, maximum oxygen uptake, and exercise tolerance) and myocardial metabolism (FDG-PET) improved, all being between 11% and 57%. Furthermore, it is noteworthy that there were no complications immediately or three months after cell transplantation, especially that there was no cardiac arrhythmia and no signs of cardiac or systemic inflammation were present.

The effects of stem cell transplantation on infarct size, cardiac function, and contractility demonstrate significant improvement of these three parameters in the therapy group (before and after stem cell therapy) as well as in the comparison between the stem cell therapy group and the control group, thus giving evidence for a beneficial therapeutic effect of stem cell therapy on cardiac performance in chronic MI.

Patients in both the stem-cell group and the control group were recruited in parallel to each other and consecutively between January 2003 and March 2004. They all (n = 36) fulfilled the same inclusion criteria. Thus, representative patient characteristics were present for the stem cell group (n = 18) and the control group (n = 18) as well as in comparing both of them. Moreover, two subsequent investigations before stem cell transplantation have been performed for each patient: investigation 1 and 2 demonstrated the stability of LV dynamics before cell therapy (9 months respectively 10 days before transplantation) and investigation 3 compared the effects of stem cell therapy with the control group. The stable hemodynamics during the preceding 9 ± 6 months before stem-cell therapy and the stable hemodynamics within the control group at all three points of investigation underline the significant alterations of the left ventriculography-derived parameters investigated after stem cell transpantation.

The regenerative potential of bone-marrow–derived stem cells may be explained by any of four mechanisms: 1) direct cell differentiation from mononuclear cells to cardiac myocytes (17), 2) cytokine-induced growing and increase of residual viable myocytes, especially within the border zone of the infarcted area (18), 3) stimulation of intrinsic myocardial stem cells (endogenous stem cells) (19,20), and 4)

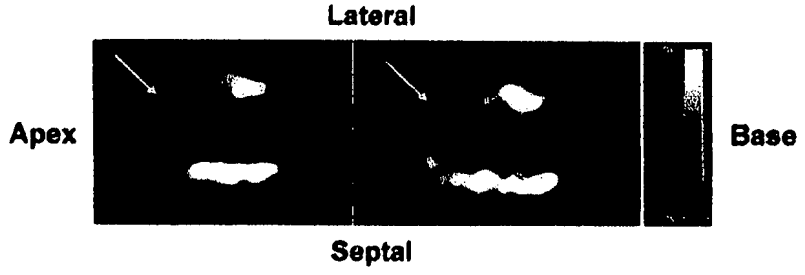

Figure 2. Representative illustration of ^{18}F-FDG-positron emission tomography (PET) before **(above)** and 3 months after **(below)** cell therapy in the transversal **(left)** and longitudinal **(right projection)** in a 30-year-old male patient with an 8-month-old anteroapical infarction. Note the restoration of glucose uptake **(below)** within the infarcted area of the formerly completely avital anteroapical myocardium.

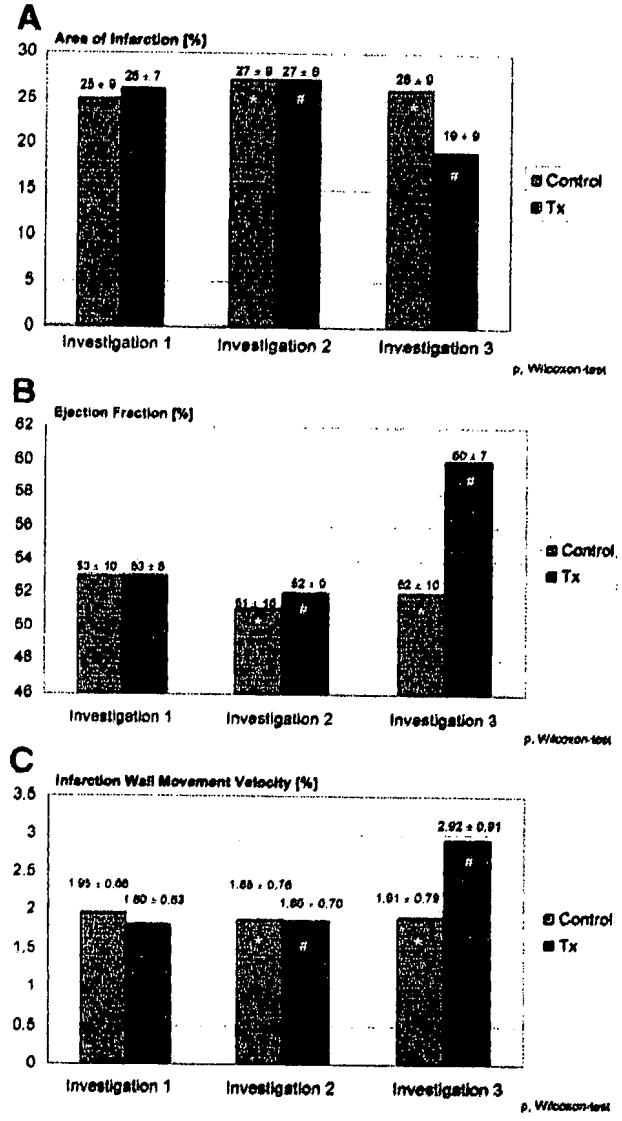

Figure 3. Illustration of the mean values of **(A)** area of infarction, **(B)** ejection fraction, and **(C)** infarction wall movement velocity, determined by quantitative left ventriculography in both groups (control group vs. transplantation [Tx] group) at the point of time: investigations 1, 2, and 3. Comparision of both groups with chronically infarcted myocardium (conrol group vs. Tx group), n = 18 patients. Investigation 1 was 9 ± 6 months before cell transplantation (controls: 9 ± 5 months before percutaneous transluminal coronary angioplasty [PTCA]); investigation 2 within 10 days before cell tranplantation (controls: at the time point of PTCA) and investigation 3 was three months after cell transplantation (controls: 8 ± 5 months after PTCA). Note the significant decrease of infarct size and the increase in ejection fraction and in contractility (infarction wall movement velocity) 3 months after cell therapy in comparison with the control group. *p = not significant (investigation 2 vs. investigation 3); #p = 0.001 (investigation 2 vs. investigation 3).

induction of cell fusion between transplanted bone marrow cells and resident myocytes (21–24).

Transdifferentiation has been described by previous investigators (4); however, it has been questioned by recent experimental studies (25). The influence of cytokines has shown to restore coronary blood vessels and muscle cells after experimental myocardial infarction. This regeneration of blood vessels and muscle cells is most pronounced in the border zone of ischemic and/or infarcted tissue (26), demonstrating an enhancement of mitotic cells and cell cycles up four-fold, when compared to areas remote from the necrotic myocardium. Moreover, mononuclear bone marrow stem cells contain a lot of cytokines (VEGF, insulin-like growth factor, platelet-derived growth factor, and so on), thereby stimulating residual normal myocytes for regeneration and proliferation and instrinsic myocardial stem cells (endogenous stem cells) for cell regeneration and for cell fusion (27–31).

Mitotic indexes are three to four times more frequent within the border zone of myocardial necrosis when compared with non-injured heart muscle (26). Moreover, 20% to 40% of intracoronarily transplanted bone-marrow–derived stem cells may be accumulated within the border zone of MI. There were no signs of apparent microcirculation disturbances because all patients had Thrombolysis In Myocardial Infarction flow grade 3. Thus, it is conceivable that in MI the border zone represents the optimum "niche" for exogenously transplanted stem cells, stimulating mitosis rates and heart muscle regeneration, preferably originating in and expanding from these areas. Cell fusion may also contribute to heart muscle regeneration, which takes its origin from the border zone, expanding gradually to the necrotic core of the infarcted area.

Our study cannot determine which cell-biologic and molecular mechanims are responsible for heart muscle repair or which of the studied factors may play the predominant role. However, the final functional outcome of this cell therapy demonstrates three main target effects: improvement in muscle function (pumping ability and contractility), myocardial perfusion (SPECT), and myocardial glucose metabolism (PET), thus giving evidence that heart muscle repair must have taken place by this intracoronary bone marrow cell transplantation procedure.

The clinical significance of this novel therapeutic approach may embrace a large number of patients with chronic coronary artery disease, preferably after previous or long-standing MI. It is conceivable that remodeling after infarction may be ameliorated or even stopped by this procedure. Thus, cell therapy may represent a new option of basic and causal therapy in chronic infarcted myocardium. It is an open question whether variations of the amount and kind of bone marrow cells, the administration technique, and the transplantation procedure itself, by enhanced environment and improvement of the angiogenic micromilieu, can further improve the milieu-dependent differentiation or regeneration of bone marrow cells in chronic infarcted heart disease. Therefore, our clinical results represent a stable basis to proceed to the next necessary step: to a larger prospective randomized study.

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Transendocardial, Autologous Bone Marrow Cell Transplantation for Severe, Chronic Ischemic Heart Failure.

Perin EC, Dohmann HF, Borojevic R, Silva SA, Sousa AL, Mesquita CT, Rossi MI, Carvalho AC, Dutra HS, Dohmann HJ, Silva GV, Belem L, Vivacqua R, Rangel FO, Esporcatte R, Geng YJ, Vaughn WK, Assad JA, Mesquita ET, Willerson JT.

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BACKGROUND: This study evaluated the hypothesis that transendocardial injections of autologous mononuclear bone marrow cells in patients with end-stage ischemic heart disease could safely promote neovascularization and improve perfusion and myocardial contractility. METHODS AND RESULTS: Twenty-one patients were enrolled in this prospective, nonrandomized, open-label study (first 14 patients, treatment; last 7 patients, control). Baseline evaluations included complete clinical and laboratory evaluations, exercise stress (ramp treadmill), 2D Doppler echocardiogram, single-photon emission computed tomography perfusion scan, and 24-hour Holter monitoring. Bone marrow mononuclear cells were harvested, isolated, washed, and resuspended in saline for injection by NOGA catheter (15 injections of 0.2 cc). Electromechanical mapping was used to identify viable myocardium (unipolar voltage >/=6.9 mV) for treatment. Treated and control patients underwent 2-month noninvasive follow-up, and treated patients alone underwent a 4-month invasive follow-up according to standard protocols and with the same procedures used as at baseline. Patient population demographics and exercise test variables did not differ significantly between the treatment and control groups; only serum creatinine and brain natriuretic peptide levels varied in laboratory evaluations at follow-up, being relatively higher in control patients. At 2 months, there was a significant reduction in total reversible defect and improvement in global left ventricular

function within the treatment group and between the treatment and control groups (P=0.02) on quantitative single-photon emission computed tomography analysis. At 4 months, there was improvement in ejection fraction from a baseline of 20% to 29% (P=0.003) and a reduction in end-systolic volume (P=0.03) in the treated patients. Electromechanical mapping revealed significant mechanical improvement of the injected segments (P<0.0005) at 4 months after treatment. CONCLUSIONS: Thus, the present study demonstrates the relative safety of intramyocardial injections of bone marrow-derived stem cells in humans with severe heart failure and the potential for improving myocardial blood flow with associated enhancement of regional and global left ventricular function.

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Milestones in the development of pediatric hematopoietic stem cell transplantation—50 years of progress

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Abstract: In the 1950s, the first infusions of hematopoietic stem cells were given as a form of treatment for childhood leukemia. This heralded the beginning of a field that has expanded to include the treatment of immune deficiencies, a variety of leukemias and solid tumors, and then genetic diseases. A number of milestones are highlighted, particularly in regard to the use of alternative sources of hematopoietic stem cells such as unrelated donors, peripheral blood stem cells and umbilical cord stem cells. In addition, newer techniques of using non-myeloablative preparative regimens helped to reduce the toxicity and long-term consequences of hematopoietic stem cell transplant. Many diseases now benefit from the replacement of the marrow stem cells and the provision of a new immune system and improved immune surveillance.

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Key words: stem cell transplantation – pediatrics – blood formation

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With the development of the age of atomic energy and interest in radiation, there developed an appreciation of the potential harm of radiation and the side-effects of exposing those in the industry to its effects, as well as the consequences of using atomic weapons. With this came an interest in understanding sensitivity to radiation and an appreciation for the hematopoietic toxicity it caused (1). In 1955–56, it was shown that mice could be protected from the lethal effects of total body irradiation with an infusion of allogeneic marrow, and in fact mice given an allogeneic marrow infusion could subsequently permanently accept a skin graft from the same marrow donor (2–4). These and other experiments showed clearly the radiation protective effect of transferring-in new cells and the development of a form of long-term chimerism and tolerance (5–10).

Abbreviations: ALL, acute lymphocytic leukemia; DLI, delayed lymphocyte infusions.

In this brief report, a number of milestones will be highlighted from the development of our current knowledge base in the field of pediatric hematopoietic stem cell transplantation (Table 1). Even the words 'hematopoietic stem cell transplantation' have evolved over time. Most work in this field of blood formation began using bone marrow. Blood as a source of peripheral stem cells was used with syngeneic transplants during the 1950s and 1960s (11, 12). However, our appreciation of the value of peripheral blood stem cells did not come about until the late 1980s and early 1990s (13). In the 1970s, the use of fetal liver tissue as a source of hematopoietic stem cells was investigated and applied in children with immune deficiencies (14). Early in their development, hematopoietic stem cells migrate from the yolk sack to the liver and temporarily make a home there before migrating to the spleen and bone marrow. Since that time in the 1980s, umbilical cord blood has been utilized as a source of hematopoietic stem cells. Motivated by the high concentration of these

Table 1. Milestones in pediatric hematopoietic stem cell transplantation

1	Experimental work
2	Initial work with children
3	Immune deficiencies
4	Selection of patients
5	Acute myelogenous leukemias
6	Neuroblastoma
7	Adding donors
8	Peripheral stem cell transplants
9	Cord blood transplants
10	Genetic diseases
11	Non-myeloablative transplants
12	Conclusions

cells during fetal development and soon after birth, the use of stem cells have been expanded *in vitro* and also been used for transplant purposes (15). More recently, the words 'stem cells' have taken on a new meaning because they describe cells which are usually obtained from early blastocysts or embryos. These cells have the capability of forming tissues or, in some cases, a whole living being. Thus, pediatric marrow transplantation has taken on new meaning and the sources of these hematopoietic stem cells have been expanded. This accounts for the change in name from pediatric marrow transplantation to pediatric hematopoietic stem cell transplantation.

Experimental work

Out of the atomic age and the atomic bomb experience came the work of the late 1940s and 1950s on protection from the hematologic effects of irradiation (16). A number of animal models were developed, and from this came the understanding that infusions of allogeneic marrow from inbred strains of mice could protect allogeneic irradiated mice (4, 8, 9). Infusions of marrow from related mice could also induce a tolerance to skin grafts (5). This led the way to an understanding of the cellular protective effects of infusions of marrow in otherwise irradiated individuals.

Initial work with children

In the 1950s, Thomas and others published their groundbreaking experience with infusions of syngeneic marrow into twins with leukemia who had been treated with supralethal doses of total body irradiation (12, 16). The prompt hematologic recovery and well being of these children against more than twice the lethal dose of irradiation attested to the protective effects of the marrow. However, these experiments took place during the initial phases of transplantation and also during the time of our initial leukemia trials, as we tried to understand how best to treat that disease. The role of continuation and maintenance chemotherapy in the treatment of childhood leukemias was not understood at all, nor were there very many chemotherapeutic agents from which to choose. As a result, by current standards these children were inadequately treated for their underlying leukemia and unfortunately had a subsequent recurrence of their underlying disease. This experience does not speak to a failure of transplantation but rather to a failure of our understanding of how to best treat leukemia back in the 1950s. It speaks to the fact that infusions of hematopoietic stem cells can provide a protective effect from the hematopoietic-suppressive effects of total body irradiation (17).

Soon after this, work began in closely related dog litter mates and this provided us with a sound scientific basis for most, if not all, of the future work to come out of the Seattle bone marrow transplant group and other groups around the world (7, 10).

Immune deficiencies

In the 1960s, a group from Minnesota used tissue typing to select a sibling marrow donor for a child with severe combined immune deficiency (14). The infusion of marrow provided immunologic recovery for this child, who is currently alive and in his 30s (14). Again, this milestone in the development of pediatric hematopoietic stem cell transplantation provided a basis for large numbers of transplants for children with a variety of immune deficiencies and set the stage for utilizing hematopoietic stem cell transplants to correct the hematopoietic and immunologic deficiencies induced by large doses of chemotherapy and radiation that are employed to treat underlying malignancies (18, 19). Although a number of other cellular and humoral factors have been utilized to treat immune deficiencies, none have been as successful as the use of hematopoietic stem cells (14, 20). Although the first child to receive such a transplant had severe combined immune deficiency and required no preparative therapy, our understanding of these many immune deficiencies has increased over time to include a realisation that some of these children require immune suppression or ablation in order to make room for a new graft or to prevent stem cell graft rejection (21). These techniques, of making a space for new stem cells resulted, in some cases, in incomplete engraftment or in

Table 2. Indications for bone marrow transplantation

Malignant disorders	Non-malignant disorders
Leukemia	Bone marrow failure syndromes
Acute myeloblastic leukemia	Acquired severe aplastic anemia
Acute lymphoblastic leukemia	Fanconi's aplastic anemia
Chronic myelogenous leukemia (adult type)	Reticular dysgenesis
Chronic myelogenous leukemia (juvenile type)	
Myelodysplastic syndromes	Immunodeficiency states
Acute myelofibrosis	Severe combined immunodeficiency disease
Some less severe combined immunodeficiency disorders	
Lymphoproliferative disorders	Wiskott–Aldrich syndrome
Hodgkin lymphoma	Some acquired immune deficiency syndrome (AIDS)
Non-Hodgkin lymphoma	
Multiple myeloma	Hematological disorders
Chronic lymphocytic leukemia	Some Thalassemia syndromes
	Some sickle cell anemias
Solid tumors	Some congenital neutropenia
Neuroblastoma	Some severe congenital platelet disorders
Bronchial carcinoma	Some Osteopetrosis
Breast carcinoma	
Melanoma	Genetic disorders
Brain tumors	Mucopolysaccharidoses
Osteosarcoma	Leukodystrophies
Ewing's Sarcoma	Other rare metabolic disorders
Teratomas	
Rhabdomyosarcoma	Connective tissue disorders
Others	Some juvenile rheumatoid arthritis
	Some systemic lupus erythematosus

others, in complete hematopoietic engraftment (14). At the present time, almost 75% of all those with severe immune deficiencies can be cured with hematopoietic stem cell transplants and the rate is even higher when a matched sibling donor is available (14).

Selection of patients

Indications for hematopoietic stem cell transplant have, to some extent, changed over the years, particularly related to the curative treatments provided for children with leukemia (Table 2) (22). In the 1980s, the most common indication for a hematopoietic stem cell transplant was salvage therapy for patients with acute lymphocytic leukemia (ALL) who failed to maintain a remission from conventional therapy. However, the likelihood of attaining long-term remission from conventional therapy is now approaching 80%, therefore eliminating the need for a marrow transplant consideration for the vast majority of such children (23). In addition, overall survival rates have also improved for children with lymphoma. Thus, the majority of children with ALL can be cured with conventional approaches and no longer require a transplant. Considering how conventional therapy has increased in intensity over time, it is true that the vast majority of children with ALL and other diseases who require a transplant have already been through a variety of therapeutic protocols and are thus more resistant to the curative effects of the preparative therapy which is provided (Table 3) (24).

Acute myelogenous leukemias

Throughout the 1980s, a number of studies suggested the utility of high dose therapy for patients with acute myelogenous leukemias (25). However, there are now available randomized results from a large-scale study of children with acute non-lymphocytic leukemia undergoing marrow transplantation with a matched sibling donor, compared to those who were treated with conventional chemotherapy because of a lack of a matched sibling donor (25). Very clearly, the long-term survival rates are higher in patients with acute non-lymphocytic leukemia who underwent transplantation with a matched sibling donor. The results from these trials would indicate that those with acute non-lymphocytic leukemia will do much better with a marrow transplant, provided there is a related matched sibling available, than with alternative forms of therapy. The recognition of this phenomena in a biologically randomized study is a key milestone in the development of hematopoietic stem cell transplantation, showing how this disease is quite

Table 3. Remaining stem cell transplant questions in 2002 for children with leukemia

1	What factors best define those to benefit from transplant therapy: (a) initial response; (b) minimal residual disease; (c) biologic markers such as chromosomes, cell morphology, cellular markers or *in vitro/in vivo* growth characteristics; (d) age and/or (e) genetic/pharmacologic profiles.
2	Are all stem cell donors alike in regards to outcome and relapse rates? Matched sibling vs. mismatched family member vs. unrelated adult donors vs umbilical cord derived stem cells.
3	Role of peripheral blood vs. marrow as a source of stem cells.
4	Methods to accelerate immune maturation of stem cell transplants or methods to minimize toxicity to the immune system.

sensitive to the preparative therapy which has been provided early in the course of the disease, as well as the immunologic effects of providing a marrow graft (25).

Neuroblastoma

Of all the solid tumors that occur in the pediatric age group, neuroblastoma has been the most resistant to conventional therapy. However, slow but steady improvement has been made in overall survival (26).

The landmark study in the development of pediatric hematopoietic stem cell transplant was the cooperative group trial which compared transplantation vs. no transplant for those with Stage IV neuroblastoma (27). Previous studies had indicated that allogeneic transplants seemed to provide no evidence for an immunologic effect of the graft itself on the underlying tumor. Fewer problems were associated with high dose therapy and autologous rescue vs. allogeneic stem cell transplant. Thus, the autologous transplant was accepted as a standard therapy for those with neuroblastoma. In fact, the current Children's Oncology Group protocol offers a course of very high dose therapy with stem cell rescue to all patients (27).

One of the major questions that remains to be decided in the next few years is whether there has been an improvement in long-term survival in those with neuroblastoma by the utilization of peripheral stem cells vs. marrow, since marrow runs the risk of contamination with tumor cells. Although most trials of autologous stem cells for transplant purposes utilized a laboratory methodology to purge neuroblastoma cells from the stem cell inoculum, the sensitivity of the assay is such that there may still be quite a number of neuroblastoma cells in the stem cell preparation that is eventually infused into patients (27). Occasionally, patterns of relapse in children following recovery of hematopoiesis suggests that there has been a miliary spread of neuroblastoma cells to a variety of tissues never previously involved with neuroblastoma, and in all likelihood, these tumor cells were contaminating the stem cell inoculum that was infused intravenously (Table 4).

Adding donors

In the late 1970s and early 1980s, a variety of clinical experiments showed the potential of applying high dose chemotherapy with hematopoietic stem cell rescue for an ever-expanding number of children and adults with malignancies, by utilizing allogeneic donors who were less than perfectly matched (28). The first group of donors to be added were those family members who were haploidentical, such as parents or siblings, or in situations where there were more extensive familial investigations to find potentially more closely matched donors (29, 30). Mismatched familial transplants are characterized by one or more major histocompatibility loci differences between donor and recipient, or complete haploidentical transplants, where there is a complete disparity at one of the two HLA A, B or DR loci, and these transplants took place in great numbers in the 1980s and early 1990s (13). Most, but not all, of these clinical protocols attempted to overcome the significant graft-vs.-host disease problems by removing more than two logs of T-lymphocytes from the donor stem cell inoculum (29, 31). From these experiments, the investigators derived a great deal of knowledge and experience in the role of T-lymphocytes when removing them from the transplant inoculum. High rates of rejection and non-engraftment

Table 4. Best approach to treatment of Stage IV neuroblastoma in 2002

1	Diagnostic biopsy with complete resection if possible.
2	Induction chemotherapy—combination—usually 5–6 cycles
3	Subsequent surgery to resect residual disease.
4	Consolidation therapy—usually high dose chemotherapy and stem cell rescue with marrow or peripheral stem cells, purged of neuroblastoma cells if present.

Table 5. Peripheral stem cells—advantages in adults

1 Easier/less painful to obtain than marrow.
2 Quicker recovery of neutrophils and platelets when used in place of marrow as a source of stem cells
3 No exposure to any anesthetic as would be necessary for collecting marrow stem cells.

suggested either that the process of removing the T-lymphocytes had eliminated extensive numbers of stem cells meant to engraft and produce hematopoietic progeny, or alternatively (28), T-lymphocytes from the donor derived inoculum were important in providing further immune suppression within the host to enable hematopoietic stem cell engraftment. To overcome the latter problem, more preparative therapy was given to some patients but this did not necessarily seem to make a difference. In addition, the removal of T-lymphocytes increased the potential for opportunistic infections due to the subsequent delay in T-lymphocyte recovery, and the delay in T-lymphocyte recovery led to an increased relapse rate in those patients receiving such marrow grafts which were T-lymphocyte depleted. However, the goal of using mismatched or haploidentical donors had achieved an important milestone which was now making it possible for every patient who had a disease that could be treated with hematopoietic stem cell transplant to be eligible for such treatment, without limitations as to whether a donor was available or not (31). The only children who failed to find a donor when considering the use of hematopoietic stem cells from mismatched or haploidentical family donors were those who had been adopted and whose families were not traceable, or alternatively children who had no siblings or parents due to some sort of environmental accident or early death.

Alongside the development of mismatched or haploidentical transplantation was the typing and recruitment of unrelated adult donors. Considering the increased knowledge of the histocompatibility loci and the further delineation of the unique HLA disparities between individuals using molecular techniques, investigations continued on typing large numbers of individuals who volunteered to register with a number of private marrow donor banks and who agreed to be called upon to donate marrow if necessary for an individual who needed a transplant and who had a tissue type similar or identical to that of the potential donor. Reports of these transplants began to appear in the 1980s and some of these were quite successful (30). As expected, most were associated with significant graft-vs.-host disease, either because our preventive treatments for GvHD were inadequate or alternatively, because there had been a lack of appreciation of minor histocompatibility differences between donor and recipient. As an increasing number of allogeneic transplants using unrelated donors developed, there also developed a national interest in bringing together all of these donor banks and setting up a single national registry supplying all allogeneic hematopoietic stem cells for transplant purposes. Thus came the government directive to set up the National Marrow Donor Program and the National Marrow Donor Registry. With increasing recruitment efforts through the 1980s and into the 1990s, well over 4,000,000 donors have been registered world-wide and listed in the NMDP registry, making it possible to find close if not perfect matches more than half the time for individuals without a matched familial donor. Several studies have documented that children will tolerate some histocompatibility difference between donor and recipient, making it possible to find unrelated donors for children with an ever increasing frequency (31). Again, another milestone had been achieved in the development of the pediatric hematopoietic stem cell field by continuing to increase the population of children eligible for transplant by defining new groups of donors, thereby circumventing the earlier problem whereby many patients could not avail themselves of marrow transplant therapy for lack of a matched related allogeneic donor.

Peripheral stem cell transplants

Although experience with syngeneic transplants had yielded the necessary information about circulating peripheral hematopoietic stem cells, to a large extent the application of this technique did not begin until the late 1980s and early 1990s (32–34). Several considerations came about to spearhead this development:

1 Some patients with malignancies undergoing high dose chemotherapy with hematopoietic stem cell reconstitution had previously received irra-

Table 6. Non-myeloablative transplant preparation

1 Reduced preparative therapy and thus potential reduced toxicity
2 Potential to eliminate all radiation.
3 Reduce growth retardation and neuro-psychological effects of full myeloablative therapy.

diation to large marrow spaces or alternatively, had a potential contamination of marrow spaces with tumor cells. Collection of peripheral blood might obviate this problem since there were few if any detectable malignant cells in the peripheral blood and thus collections of stem cells in the peripheral blood would theoretically be less likely to be contaminated with tumor cells.

2 Going to the operating room to obtain hematopoietic stem cells from the marrow space was traumatic and painful, and collections of peripheral blood stem cells could be done as an outpatient in a less painful setting with far less morbidity (33).

As a result, many studies were published on the utilization of peripheral stem cells on how best to collect them and on the timing of collection (32–34). With the development of hematopoietic stem cell cytokines stimulating the production of CD34+ cells, there was now an understanding of how best to improve the yield of collected stem cells. Subsequent studies showed that peripheral stem cells, when given to a host who had undergone ablative chemotherapy or chemotherapy plus radiation, resulted in faster hematopoietic engraftment, fewer hospital days, and potentially improved immune function more quickly than when using hematopoietic stem cells from the marrow space (34). Although similar studies have not been done in children due to the numbers of children available for such studies, in some diseases we have already accepted the utility of obtaining peripheral blood stem cells for transplant purposes (33, 35, 36). However, in many situations in pediatrics, the donors are small children and it therefore becomes more difficult to collect peripheral stem cells from them and more likely that we can obtain an equal or higher total number of hematopoietic stem cells by taking the donor to the operating room and obtaining bone marrow in the traditional manner (33). Thus, even though the use of peripheral stem cells was first documented in pediatric transplants more than 30 years ago, when blood from one twin was infused into another (11), the large scale use of peripheral stem cells, the appreciation of the fact that the stem cells collected from the peripheral blood were somewhat different from those collected from the marrow space, and the extension of this technique to those who have potential marrow involvement with tumor was a whole new era and marked a new milestone in the further development of pediatric hematopoietic stem cell transplantation (Table 5) (32, 33).

Cord blood transplants

In the 1970s, the migration of hematopoietic stem cells from the yolk sack to the liver was understood to the extent that fetal livers were obtained from aborted fetuses and successfully used for transplant purposes (37). Over a period of 10 years, there developed an appreciation of the vast numbers of hematopoietic stem cells in the developing fetus, which at the time of birth is more than 100-fold greater per volume of blood than that found in adults. The sense was that the developing fetus was rapidly growing and needed ever increasing quantities of hematopoietic and immunologic cells. This was translated into an increased number of hematopoietic stem cells, developing in a naive individual and incapable of recognizing and rejecting maternal tissue and probably having little capacity to reject other tissue soon after birth. We knew from hundreds of years of clinical observation that infants were highly susceptible to a wide variety of infectious problems, only because of the lack of immunity at birth and that there was a normal maturation of the immune system in the first 1–2 years of life. In fact, it was well known that children with immune deficiencies did not usually present within the first few months of birth because of the protective levels of cellular and humoral immune factors that are passed from mother to fetus, and that when this protection eventually waned, then the infant's immunodeficiency was exposed (20). With this background in mind, there developed an appreciation of the idea of obtaining umbilical cord blood at the time of birth and using it as a rich source of hematopoietic stem cells which could then be used for transplant purposes (15, 38). Several significant advantages immediately became apparent:

1 With millions of births worldwide, it should be possible to collect umbilical cord blood from every child soon after birth and such blood/cells could be typed and cryopreserved and made available to any individuals with a similar tissue type.

2 Unlike the situation where an adult allogeneic donor needs to be taken to the operating room to obtain marrow or needs to visit a blood bank in order to provide peripheral blood stem cells, the use of these umbilical cord cells entailed no harm to the donor since these blood cells were usually discarded with the placenta at the time of birth.

3 Because the umbilical cord-derived hematopoietic stem cells were naive, it should be possible to use them more readily in situations where there was not a perfect HLA match between donor and

recipient and still see a good hematopoietic engraftment without the lethal complications of graft-vs.-host disease.

In 1988, the first such transplant took place in a child with Fanconi's anemia, receiving hematopoietic stem cells from a previously collected umbilical cord blood specimen from a sibling who was perfectly matched (39, 40). Since that time, a number of umbilical cord blood banks have been set up around the world, some linked by computer and some not, but all dedicated to the business of providing umbilical cord blood for transplant purposes, particularly in situations where there were no available matched sibling donors (15). These transplants have continued in select centers around the world and to date, there still are some problems and difficulties:

1 Umbilical cord-derived hematopoietic stem cells tend to grow a little more slowly, perhaps related to their concentration and numbers following cryopreservation, than freshly obtained peripheral blood stem cells or marrow stem cells from adult donors. The slowness to recovery of blood counts may predispose the transplanted patient to an increased number of infectious problems and complications (38).

2 Although one of the advantages of using hematopoietic stem cells derived from the umbilical cord is their relative naiveté and the potential for a lack of graft-vs.-host disease, there developed a sense in the 1980s and 1990s of the importance of graft-vs.-host disease in providing an immunologic method for controlling the subsequent recurrence of a malignancy following high dose preparative therapy. The absence of such graft-vs.-host disease following the transplant of hematopoietic stem cells derived from umbilical cord predisposes some patients to an increased risk for recurrence of their underlying leukemia (15); and

3 The amount of cord blood obtained in the delivery room is directly related to the experience of the operator. The amount of cord blood collected correlates with the number of stem cells and the number of stem cells in a sample used for transplant purposes correlates with the rate of engraftment. The rate of engraftment and the number of stem cells appears to correlate with ultimate survival (41). Thus, there are many specimens of umbilical cord blood obtained in the delivery room which are low in volume and which are therefore probably not well suited for use in larger children or adults (38).

The availability of umbilical cord blood has once again been a milestone because it has enabled more children who otherwise could not find a suitable donor to have one available. In addition, many children undergoing marrow transplantation need not suffer graft-vs.-host disease and its attendant morbidity/mortality, such as those with genetic diseases or immune deficiencies (40–42). As a result, cord blood has then become an ideal source of hematopoietic stem cells. The first patient transplanted with umbilical cord blood as previously mentioned was a child with Fanconi's anemia, and there had not yet been any evidence of myelodysplasia or leukemic changes (39, 40). As a result, the child would not theoretically benefit from the occurrence of any graft-vs.-host disease and thus the use of umbilical cord blood as a source of hematopoietic stem cells was an ideal source in that particular situation. The overall place of umbilical cord blood in the field of hematopoietic stem cell transplantation remains to be determined, but clearly the advent of this technology and the establishment of numerous umbilical cord blood banks has been a major milestone in the field of pediatric hematopoietic transplantation.

Genetic diseases

For some patients, hematopoietic stem cell transplants are a very crude way of providing new genetic material. It is not selective but rather provides a host of hematopoietic and immunologic stem cells, all of which may bring missing enzymes or missing substrates not present in a child undergoing such a transplant for what is presently defective. Two such examples are noteworthy.

Almost 20 years ago, the first marrow transplant took place in a child with sickle cell anemia (42). Sickle cell anemia is an inherited genetic disorder characterized by abnormal hematopoiesis, deformities and increased sickling of red cells secondary to abnormal hemoglobin synthesis and an associated array of clinical difficulties. The replacement of these abnormal hematopoietic stem cells with new ones making cells with normal hemoglobin eliminates future problems associated with the disease for the child undergoing such a transplant (43). In fact, hematopoietic transplants have been curative (40). The problem is related primarily to the selection of patients: when to offer the transplants to patients and when too much damage may have been done by the underlying disease to warrant proceeding with the transplants. Since the vast majority of children with sickle cell disease make it into adult life, families have a very difficult time

making a decision to proceed with a marrow transplant when there might be as high as a 15–30% significant morbidity and mortality associated with the procedure (44, 45).

A number of mucopolysaccharide disorders, as an example of another genetic disorder, have been cured with a hematopoietic stem cell transplant (46). In these cases, there is a missing enzyme which can be found within neutrophils and other cells originating from the bone marrow space, and these new cells will replace the missing enzyme and deal with substrates which accumulate in abnormal places where such accumulation may result in significant damage. In most of the mucopolysaccharide disorders, proteins accumulate in the nervous system as well as in the heart and liver and eventually lead to the dysfunction of these organs and death. A marrow transplant provides enzymes which are missing to help degrade and deal with the substrate which accumulates. Damage previously associated with the abnormal condition may not be repaired over time but in general, one is looking to halt the progression of the underlying disease.

Significant progress has been made in learning for which diseases it is best to offer such transplants, the appropriate timing, and the limitation of the procedures in other patients in terms of correcting or halting the further progression of the underlying illness.

Non-myeloablative transplants

It has been well known for some time that one of the beneficial aspects of an allogeneic stem cell transplant has been the immunologic surveillance and immunologic treatment which is brought about by the growth of a new immune system. Patients experience graft-vs.-host disease with variable frequency and graft-vs.-host disease has been shown in several studies to be helpful if not essential in preventing the recurrence of an underlying malignancy. There appears to be a graft-vs.-tumor or graft-vs.-leukemia effect from the new source of allogeneic hematopoietic stem cells (47, 48).

This fact was further emphasized by studies using delayed lymphocyte infusions (DLI) to treat recurrent disease, as well as to treat significant viral infections which occur post-transplantation (47–49). Considering the delay in the recovery of normal T-lymphocytes following an allograft, lymphocytes can be obtained from living hematopoietic stem cell donors and these cells can be utilized for treatment, either to prevent recurrent diseases by prophylactic infusions of such lymphocytes or to treat the recurrent chronic myelogenous leukemia which may occur following a transplant.

Considering the immunologic effects of the graft, and animal work which showed that with minimal conditioning therapy and minimal establishment of an allograft within the bone marrow space, further immune suppression from donor lymphocytes would effectively create complete chimerism and full engraftment, thereby providing individuals with the benefit of the new immune system provided with the new allograft (50). This approach holds great promise in establishing the presence of a new allograft and as such is really the goal of the transplant process and at the same time, limit the preparative therapy given, since a complete establishment of hematopoiesis is not initially necessary but will be effected by subsequent infusions of lymphocytes. This has been one of the milestones in the development of pediatric hematopoietic stem cell transplantation. Those with an enzyme disorder, and potentially those with disorders only characterized by missing cellular function, need a small proportion of hematopoietic stem cells to fully engraft and function to correct the underlying defect (50). A recent patient reported with Chédiak Higashi syndrome is a good example whereby only a small proportion of the neutrophils are actually of donor origin and therefore normal, but the actual number is high enough to have prevented any significant infections occurring in the years following the establishment of the allograft (51).

The use of these non-myeloablative transplants, meaning that the preparative therapy has been limited in extent and toxicity, has permitted a very small proportion of donor cells to engraft, but these cells are then engineered to become fully chimeric with the recipient by the use of DLI to further suppress the host. These types of transplants, whereby the preparative therapy is minimized, are undergoing trials in the elderly and in more fragile individuals who would otherwise not qualify for transplant, in the hope that they will receive the beneficial effects of a new immune system and the immunologic surveillance thus provided, without the toxicity which often is associated with the preparative therapy given to bring about full engraftment. In addition, there are children with disorders that would potentially be made worse with full doses of preparative therapy, including total body irradiation. Thus a non-myeloablative transplant limits the amount and type of preparative therapy given, thereby limiting the potential toxicity to

the patient. Limiting the preparative therapy reduces the long-term effects but still permits full eventual engraftment and provision of the missing components of the immune system or immune surveillance (Table 6).

Conclusions

When hematopoietic stem cell transplants began almost 50 years ago, they were a shot in the dark to try and correct underlying significant diseases and were eventually utilized to correct immune deficiencies. Over the ensuing 40 years, a variety of newer techniques were developed to perfect the source of stem cells and increase our understanding of clinical situations in which one source of stem cells might be better than another (52, 53). Improvements in supportive care made it possible for an ever increasing number of children to survive the effects of the preparative therapy and the subsequent graft-vs.-host disease and immaturity of the immune system. High dose therapy with hematopoietic stem cell rescue has become a mainstay of modern therapy for children with Stage IV neuroblastoma and a significant salvage therapy for patients with a variety of other diseases when these children have failed to respond to more conventional approaches. The approaching frontier involves the *in utero* identification of genetic defects and immunodeficiency diseases, thereby utilizing *in utero*-administered hematopoietic stem cells to provide definitive curative therapy (54, 55). Although children with leukemia make up an ever decreasing number of those eligible for transplantation because of the success of the initial therapeutic non-transplant treatment they receive (22), there still are a number of children benefiting from transplantation who have been treated rather heavily with alternative therapy and we know that there are a variety of diseases that now benefit from replacement of the marrow graft and provision of a new immune system and improved immune surveillance.

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Automated isolation of mononuclear cells using the Fenwal CS3000 blood cell separator.

Areman EM, Cullis H, Sacher RA, Cottler-Fox M, Deeg HJ.

Division of Transfusion Medicine and Bone Marrow Transplantation, Georgetown University Hospital, Washington, D.C.

We describe a method for in vitro isolation of mononuclear cells from peripheral blood or bone marrow using a Fenwal CS3000 Apheresis device without employing density gradients or sedimenting agents. The automatic processing program requires minimal operator intervention and no subjective operator decisions. A mean of 67% of starting mononuclear cells were recovered in a 100 ml product having 95% mononuclear cells and less than 1% of the original red blood cells. The average processing time was 35 minutes.

PMID: 2308992 [PubMed - indexed for MEDLINE]

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Human bone marrow processing using Cobe 2991 and CS 3000 blood cell separators for further ex vivo manipulation.

Angelini A, Dragani A, Iacone A, D'Antonio D, Accorsi P, Quaglietta AM, Berardi A, Fioritoni G, Di Bartolomeo P, D'Emilio G, et al.

Divisione di Ematologia e Centro Trasfusionale, Ospeda-le Civile, Pescara, Italy.

Several automated procedures are now available to enrich stem cells from large bone marrow (BM) volumes prior to ex vivo treatment or cryopreservation. This report details our experience using a ficoll-hypaque (F/H) gradient on Cobe 2991 cell washer and a CS 3000 continuous flow separator, on 90 BM processed for allogeneic and autologous transplantation. In the Cobe series, from 70 BM aspirates, 89 +/- 5% of the original mononuclear cells (MNC) was found in the light density fraction with a erythrocytes (RBC) and granulocytes (PMN) removal of 98 +/- 1 and 97 +/- 4.5%, respectively. Over 80% of the initial myeloid precursors (CFU-GM) were recovered in a small final volume. Twenty BM processing were performed with a CS 3000 separator using program "3" and granulo chamber. This technique yielded 86 +/- 9% of the initial MNC while 85 +/- 10% of RBC and 90 +/- 1.1% of PMN was removed. Over 75% of the original CFU-GM was recovered in the final product. Both techniques are effective to large-scale purification of progenitor cells and readily available as routine procedures for marrow processing.

PMID: 2351343 [PubMed - indexed for MEDLINE]

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1: J Hematother. 1992 Winter;1(4):349-59. Related Articles, Links

Use of the Terumo SteriCell for the processing of bone marrow and peripheral blood stem cells.

Janssen WE, Lee C, Smilee R, Carter R.

University of South Florida, Department of Internal Medicine, Tampa.

The SteriCell cell processing instrument is a good choice for a stem cell processing laboratory that is of sufficient size that they cannot share an apheresis machine with the blood bank. It is a laboratory instrument, with no facility for patient connection. Because of its minimal size and weight, it is easily stored in a cramped laboratory. Its automated programs are appropriate for processing of bone marrow and peripheral blood stem cells, and it is quite easy to learn how to use (in our laboratory, most individuals have been completely facile with the SteriCell after fewer than six processings). Based on reported results from other instruments, the SteriCell provides cell yields that are comparable to competing instruments. Service (provided by Haemonetics) has been satisfactory, and support from Terumo has been excellent. We can recommend this instrument to any other laboratory.

PMID: 1345677 [PubMed - indexed for MEDLINE]

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EXHIBIT E

Caplan 1991 publication
Journal of Orthopaedic Research
entitled "Mesenchymal Stem Cells"

Journal of Orthopaedic Research
9:641–650 Raven Press, Ltd., New York

Mesenchymal Stem Cells*

Arnold I. Caplan

The Skeletal Research Center, Department of Biology, Case Western Reserve University, Cleveland, Ohio, U.S.A.

Summary: Bone and cartilage formation in the embryo and repair and turnover in the adult involve the progeny of a small number of cells called mesenchymal stem cells. These cells divide, and their progeny become committed to a specific and distinctive phenotypic pathway, a lineage with discrete steps and, finally, end-stage cells involved with fabrication of a unique tissue type, e.g., cartilage or bone. Local cuing (extrinsic factors) and the genomic potential (intrinsic factors) interact at each lineage step to control the rate and characteristic phenotype of the cells in the emerging tissue. The study of these mesenchymal stem cells, whether isolated from embryos or adults, provides the basis for the emergence of a new therapeutic technology of self-cell repair. The isolation, mitotic expansion, and site-directed delivery of autologous stem cells can govern the rapid and specific repair of skeletal tissues. Key Words: Mesenchymal stem cells—Bone—Cartilage—Differentiation—Self-cell therapy—Skeletal tissue—Embryo—Adult.

THE CONCEPT

It is generally agreed that in an embryo a mesenchymal stem cell is a pluripotent progenitor cell which divides many times and whose progeny eventually gives rise to skeletal tissues: cartilage, bone, tendon, ligament, marrow stroma, connective tissue (Fig. 1). By definition, these stem cells are not governed by or limited to a fixed number of mitotic divisions. Their progeny are affected by a number of factors, however, as they become tracked into very specific developmental pathways in which both intrinsic and extrinsic factors combine to control the molecular and cellular pattern of expression that results in specific tissues that perform specific functions based on their molecular repertoire (9,11).

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* This publication was, in part, the basis for Dr. Caplan's receiving the Elizabeth Winston Lanier Award given by the American Academy of Orthopaedic Surgeons as part of their 1990 Kappa Delta Awards.

Indeed, the progression from stem cell to final end phenotype is marked by discrete stages with transit from one stage to the next dependent on local cuing from surrounding cells (paracrine regulation) as well as signals emitted by the cell itself and the reception of its own signaling (autocrine regulation) (10,57). The sum of these various intrinsic and extrinsic signals defines the developmental position of the cells. Although difficult to reconstruct on a cell culture dish, such "positional information" has been experimentally approached by studying embryonic cells in culture, cells that have the potential to differentiate into various phenotypes (7,9,11,15).

The concept of stem cells is now well established (21,60). Two systems serve as models for such a concept: First, *Caenorhabditus elegans* is a small worm whose entire developmental lineage map has been described (21); every cell found in the adult has been carefully tracked and its progenitor tree precisely established with every branch and subbranch delineated. Second, and to be emphasized, the heamtopoietic cell lineage has been described with its several diverging pathways (21,52). It is now clear that each separate pathway and, indeed,

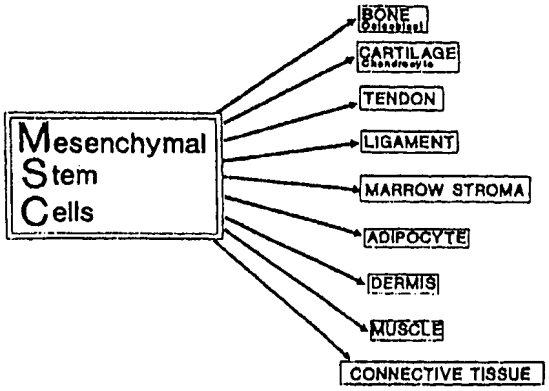

FIG. 1. Mesenchymal stem cell phenotypes. Mesenchymal stem cells are theoretically capable of differentiating through a series of separate and unique lineage transitions into a variety of end-stage phenotypes as shown.

progression through each separate stage within a discrete pathway is controlled by a balance of extrinsic and intrinsic macromolecules. Molecular biologists continue to isolate, clone, and express large amounts of these proteins, which allows use of cell culture systems to identify accurately the factor that controls progression to which stage and when (51,70). The challenge for skeletal biologists is to use the new information and new molecular tools to translate these advances into a better understanding of skeletal development, physiology, and repair.

EMBRYONIC MESENCHYMAL CELLS

The middle embryonic layer, the mesoderm, gives rise to all of the body's skeletal elements.* The term, mesenchyme, is derived from the Greek meaning "middle" (meso) "infusion" and refers to the ability of mesenchymatous cells to spread and migrate in early embryonic development between the ectodermal and endodermal layers. This characteristic migratory, space-filling ability is the key element of all wound repair in adult organisms involving mesenchymal cells in skin (dermis), bone (periosteum), or muscle (perimysium). Proteins that serve as chemoattractants, chemicals that specifically encourage this migratory activity to wound or developmental sites have been identified (24,32,59). The migratory activity of mesenchymal cells is complemented by their capacity to aggregate specifically to form unique developmental structures or, in adults, to form repair blastemas, which are then capable of responding to local cues and differentiating accordingly to achieve regenerative repair (10,11).

* For the sake of clarity, I address only issues related to cartilage or bone, although the same general experimental approach and logic can be used for other mesenchymal tissues.

Chick Limb Cells

More than 20 years ago, my collaborators and I attempted to define experimentally the conditions and cues necessary to control the differentiation of embryonic mesenchymal cells into cartilage and bone (5,7,17). Both in vivo and in vitro studies were used, but development of cell cultures and the general approach of using cell cultures has provided the experiential basis for approaching the study of mesenchymal stem cells from adults. The system we developed was the culturing of stage 24 (day 4.5) embryonic chick limb mesenchymal cells under conditions that promoted differentiation of cartilage (chondrocytes) (5,7,13,20) and bone (osteoblasts) (42,65).

Chondrocytes

Our first experimental effort with embryonic chick limb mesenchymal cells was to focus on chondrocyte development, which we learned was controlled by the initial plating density (5,17), oxygen levels (14), or, as recently shown by other investigators, a variety of physical and chemical factors (53,58,61). The key factor in the conversion of a mesenchymal cell to a chondrocyte is maintaining the progenitor cell in a round, unspread conformation. This can be accomplished simply by plating the cells initially under very compact, high-density conditions: 5×10^6 embryonic stage-24 limb mesenchymal cells per 35-mm dish (5,17). Even in a simple, defined medium consisting of insulin, transferrin, bovine serum albumin (BSA), and hydrocortisone in Eagle's minimum essential medium (MEM), the differentiation of chondrocytes and their further development can be documented as long as the cells are initially seeded at high density (18,30).

The high-density, limb cell-derived chondrocyte in culture makes two cartilage-specific molecules in abundance: type II collagen (68) and a large chondroitin sulfate, keratan sulfate proteoglycan (CSPG) (13,18,20). By detailed chemical and physical characterization of the CSPG synthesized on each day of culture, we showed that the glycosaminoglycan chains are biosynthesized slightly differently with

time (Fig. 2). Peptide maps show that the newly synthesized core protein (26) is identical on each day of culture, whereas the chondroitin sulfate chains are synthesized progressively shorter (30,000 D on day 2 to 15,000 D on day 20) and the keratan sulfate chains are synthesized progressively larger (0 to 10,000 D) (13,20). This biosynthetic progression is exactly what has subsequently been shown to occur in the cartilages of embryonic, adult, and aging human (50) and bovine specimens (62).

That embryonic chondrocytes have an aging-dependent program of changing biosynthesis is further documented when cultured embryonic chick chondrocytes are transplanted in a fibrin-based delivery vehicle into defects at the articular surface of adult chickens (29). Such chondrocytes produce what appears to be appropriate cartilaginous matrix and have been followed >18 months. The resulting repair cartilage appears to integrate perfectly into the defect and to provide the animal with a healthy, normal articular surface. These experiments and others clearly establish the concept of repairing cartilage with embryonic or appropriate reparative cells.

Osteoblasts

Our initial success in studying emergence of chondrocytes and formation of cartilaginous tissue from cultures of limb mesenchymal cells encouraged us to study differentiation of osteoblasts and formation of bone as well. Our initial logic was that high-density conditions caused cartilage formation and that cartilage was the progenitor tissue of bone. (Some investigators have reported that cartilage provides the scaffold for bone formation.) After 2 years of frustrating experimentation, we realized that when infrequent bone and osteoblasts could be identified, the bone had formed at a distance from cartilage and never on or in the cartilage (42). By carefully decreasing the initial cell density of limb mesenchymal cells to just below the density at which some mineralized cartilage could form (2×10^6 cells/35-mm dish), we observed numerous deposits of bone and abundant osteoblasts which, again, were clearly at some distance from cartilage (6,42,44). In addition, these osteoblasts exhibited the classic response to parathyroid hormone (PTH) of elevated cyclic AMP levels (71,72) and possessed a bone-specific alkaline phosphatase (43). These studies clearly indicated that embryonic chick limb mesenchymal cells were capable of differentiating into osteoblasts and that the culture conditions supporting optimum osteoblast emergence were different from the conditions optimum for chondrogenesis.

Mouse and Human Limb Cells

With regard to cartilage and bone, the properties of mouse and human limb mesenchymal cells in culture appear to be quite similar, if not identical (25,46). Likewise, cartilage and bone development in vivo are also quite comparable, with the major exception that embryonic cartilage of chick does not calcify whereas that of mammals always calcifies (16). The comparable developmental properties of aves, rodents, and humans encourages us to continue experimentation with animal cells as an approximation of better understanding of the properties of human cells and tissues.

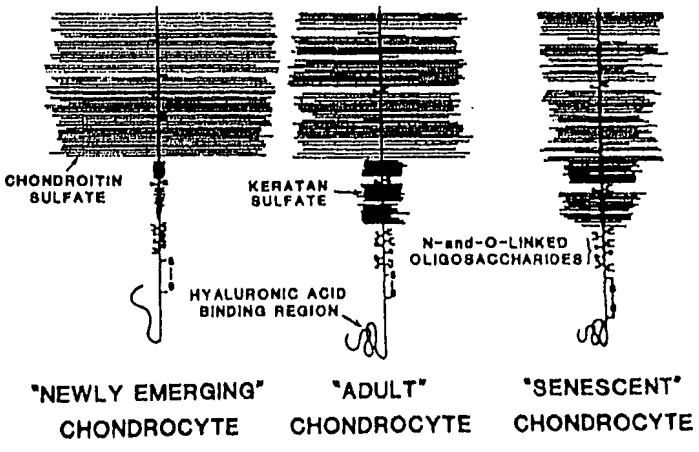

FIG. 2. Proteoglycans synthesized by newly differentiated, mature, and senescent chondrocytes. With increasing age, chondrocytes synthesize proteoglycans that have smaller chondroitin sulfate chains and larger keratan sulfate chains (7,8,12,13,20).

LINEAGE OF MESENCHYMAL CELLS

Cartilage

The important inference from the above discussion is that chondrocytes have a programmed (i.e., genetically dictated) sequence of changes in their end-stage expression (8,12). The differences in glycosaminoglycan chain lengths or chemistry are stable to cell culturing or metabolic perturbation. The control of these events is not known, but all experiments designed to slow this sequence of biosynthetic alterations or reverse them have failed. The inference is that a genomic mechanism somehow "tells time" and that this clock is hard-wired and unidirectional (8,12).

Such biosynthetic changes in articular cartilage are different from the lineage changes observed in adult growth plate or embryonic limb cartilage. A discrete set of expressional stages or lineage states, comprising dividing, maturing, and hypertrophic chondrocytes, is apparent in embryonic limb tissue, cell culture (13,58,61), and in the growth plate (19,28). Eventually, the hypertrophic cartilage in vivo is eroded by vascular, marrow, and phagocytic cells and replaced by bone. Each chondrocytic lineage state is uniquely different from its predecessor, as shown in Fig. 3. For example, hypertrophic chondrocytes synthesize a unique small collagen, type X, and a unique proteoglycan (54,55); neither of these molecules is synthesized by mature chondrocytes. In this particular circumstance, several factors are proposed to contribute to conversion of mature chondrocytes to hypertrophic chondrocytes (35); reversal of this process has not been reported.

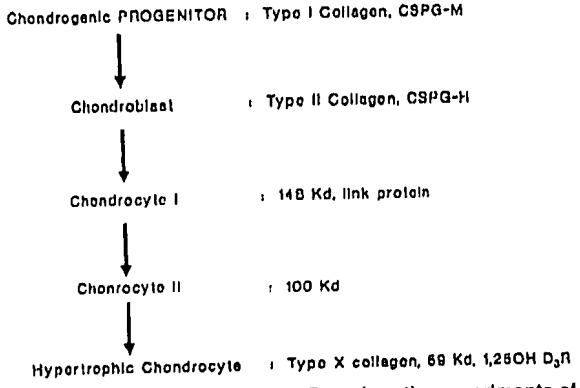

FIG. 3. Chondrogenic lineage. Based on the experiments of Solursh et al. (58,61) a hypothetical lineage map can be constructed to consist of at least five separate stages based on the changing biosynthesis of proteins (named or by molecular weight, K_d) or chondroitin sulfate proteoglycan (CSPG). The receptor for 1,25-dihydroxy Vitamin D_3 is represented as $1,25OHD_3R$.

Bone

We recently reviewed the major aspects of embryonic bone development. Figure 4 shows several important elements or rules governing this complex process (10,11,16). First, a discrete positioning of progenitor cells, stacked cells, existed in proximity to the developing bone (47). The stacked cells give rise to osteoblats in a discrete series of lineage steps (described below). The end stage or secretory osteoblast is positioned by its proximity to vasculature, with the "back" of the osteoblast to the capillary and osteoid deposited from the "front" of this highly oriented secretory cell (47,48). The vasculature is the orientor of osteogenesis and the osteoblast is the formative element. Cartilage is not replaced by bone, but is instead the target for vascular (marrow) replacement (48); in the early limb, the cartilage model exactly defines the eventual marrow cavity.

That a discrete series of individual lineage stages exists between the progenitor cells in the stacked cell layer and the secretory osteoblasts is now clear, as shown in Fig. 5. We recently isolated four monoclonal antibodies, SB1, 2, 3, and 5, which have helped provide evidence for an osteoblast lineage (3,4). Progenitor cells in the stacked cell layer and osteocytes do not interact with SB1, 2, or 3. Newly differentiated osteogenic cells react with SB1, but not with SB2 or 3, whereas fully secretory osteoblasts react with SB1, 2, and 3. A subpopulation of osteogenic cells reacts with SB2, but not SB3. Osteocytes react with OB7.3 of Nijweide and Mulder (38) or with our SB5, but not with SB1, 2, or 3. The lineage tree in Fig. 3 is based on these observations and not only establishes the existence of an osteoblastic lineage but suggests that osteocytes are derived directly from osteoblasts with SB1, 2, and 3 antigens that are suppressed as SB5 and OB7.3 are turned on. Experiments are now in progress to use these monoclonal antibodies to isolate representatives of each lineage stage so that studies can be conducted to identify the agents that promote the progression from one lineage stage to the next. Central to the thesis presented below is the existence of osteoprogenitor cells in the stacked cell layer, the future periosteum.

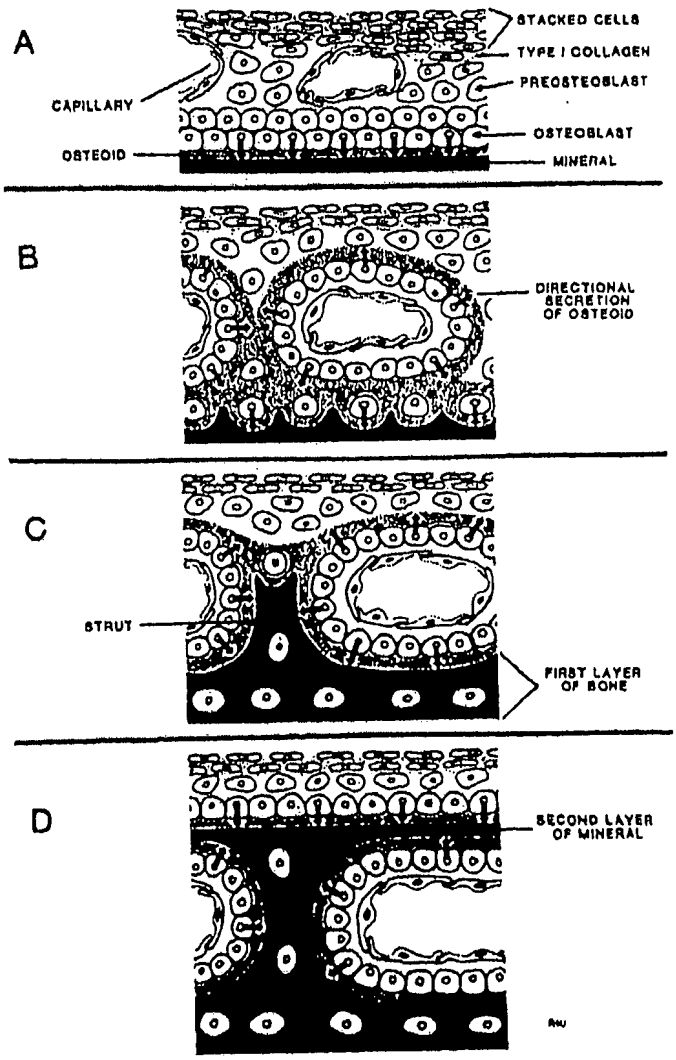

FIG. 4. Sequence of progressive in vivo bone development. Progressive repositioning of the vasculature from outside the stacked cell layer to a position in close approximation to the first layer of secretory osteoblasts responsible for formation of the first bony collar of the chick tibia (11,47,48). The osteoblast is oriented with its back toward the invading capillary and secretion of osteoid toward the cartilage core from the osteoblast's face. In this model, osteoblasts secrete osteoid in a direction away from vasculature (B), causing formation of a strut (C) and eventually forming the second layer of bone (D). These observations show that an intimate relationship exists between vasculature and newly forming bone.

BIOACTIVE FACTORS IN BONE

From the earliest days of modern humans, bone has been recognized to have the powerful capacity to repair discontinuities (22). A variety of bioactive factors combine in a complex multicellular, multistep response in which reparative cells are specifically attracted to the repair site. These cells then aggregate, multiply, bridge the bone gap, and differentiate into chondrocytes or osteoblasts as controlled by the proximity to vasculature. Recently, an intensive research activity to identify and characterize these various bioactive factors was largely successful (56,66,67,69). Our laboratory has described the purification of a protein factor, chondrogenic stimulating activity (CSA), which converts embryonic limb mesenchymal cells to chondrocytes (63,64). We are also attempting to purify a bone-derived chemoattractant for mesenchymal cells by using the now standard modified Boyden chamber (31,33).

Relevant to the thesis developed below, the identity and manipulation of the cells responding to bone-derived bioactive factors is directly related to successful bone repair. Such responding cells are present in the adult periosteum (36), dermis (49),

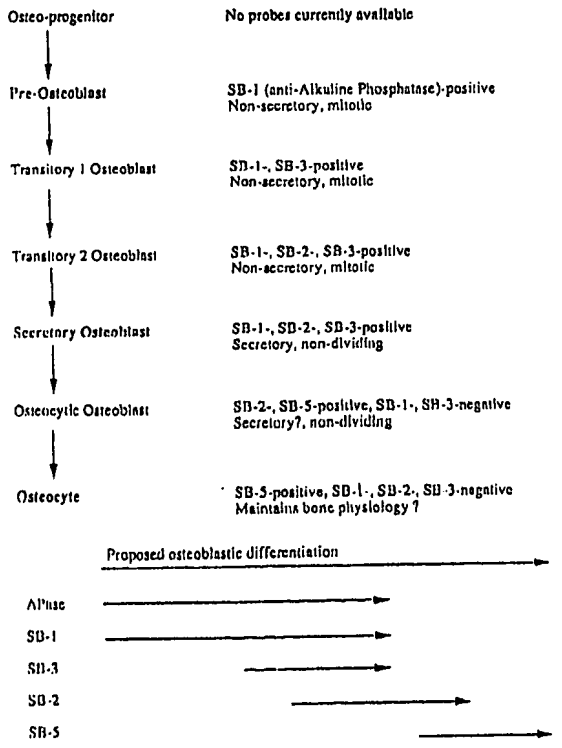

FIG. 5. Osteogenic cell lineage. Based on recent experimentation in which monoclonal antibodies were generated to cell surface antigens of osteogenic cells (3,4), a tentative lineage diagram reflects acquisition or loss of specific antigenic determinants. The characteristics of SB1, 2, and 3 were described previously (3); SB-5 (4) has been characterized and is similar to OB7.3 of Nijweide and Mulder (38). The individual lineage states are not weighted in terms of their prevalence or dwell-time; e.g., "transitory osteoblast 1" occurs rarely and cannot be recognized easily except at specific times and locations, whereas the "secretory osteoblast" is easily recognized and plentiful.

bone marrow (1,40,41,45), and connective tissue associated with muscle (34,37). One or all of these repositories are capable of forming bone when appropriately delivered bioactive factors are presented.

Alternately, when the responsive cells, stem cells, are placed in suitable delivery vehicles that can retain these cells while encouraging vascular invasion, bone can be observed to form. Recently, we used calcium phosphate porous ceramics in composite with marrow to encourage bone formation at both heterotopic and orthotopic sites (40,41). Whole disaggregated marrow cells in suspension are loaded into porous ceramic and transplanted to subcutaneous, intramuscular, or bone defect sites in vivo. In 1–2 months, the few mesenchymal stem cells in the marrow have replicated massively and differentiated into osteoblasts. In the dead-end pores of the ceramic, which are devoid of vasculature, these stem cells differentiate into chondrocytes and form cartilage.

MESENCHYMAL STEM CELLS

From the above discussion several key facts are evident. First, embryonic mesenchymal stem cells in the limb which give rise to cartilage and bone in vivo can be manipulated in vitro. Second, these cells have a lineage progression of separate, individual steps, whether it be the chondrogenic or osteogenic pathway. Third, local cuing, sometimes involving highly potent protein factors, is responsible for providing positional information and causing lineage progression. Cell culture conditions have been refined to the extent that not only can these progressive events be studied in detail, but manipulation of the cells is also possible to provide control of tissue size and function.

Fourth, although chondrocytes and osteoblasts are derived from a common mesenchymal cell, the conditions for their initial differentiation and progression through the individual steps of their lineages are uniquely different. For example, osteogenesis is dependent on proximity to vasculature whereas chondrogenesis requires the complete absence of vasculature (7,10,11,16); osteogenesis is optimum at an initial cell culture seeding density in 35-mm dishes of 2×10^6 embryonic limb mesenchymal cells, whereas chondrogenesis is optimum at 5×10^6 cells (5,17,42).

Fifth, bone forms from mesenchymal stem cells in a cartilage-independent manner with vasculature providing a determinative discriminator between these two tissues; embryonic cartilage is not replaced by bone, but rather by vasculature and marrow (10,11,16). Sixth, we can demonstrate that three tissue sites are the repositories of mesenchymal stem cells: marrow (1,40,41,45), periosteum (36), and muscle connective tissue (34,37).

MARROW

Figure 6 outlines an assay to demonstrate that marrow contains mesenchymal stem cells capable of differentiation into cartilage and bone. Whole marrow is disrupted into single cells by passing it through needles of successively smaller sizes; the

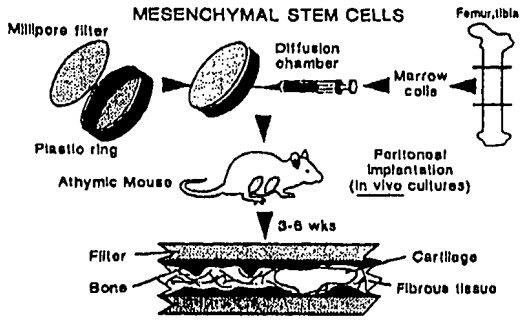

FIG. 6. Diffusion chamber assay in nude mice. Cell samples from marrow or other sources can be loaded into chambers composed of two Millipore filters glued to the edges of a plastic ring. These chambers are then implanted in the peritoneal cavity of athymic (nude) mice as a highly vascular in vivo incubation site. The filters prevent host cells from entering the chambers but permit rapid diffusion of nutrients and other factors into or out of the chamber. Histologic identification of two distinctive phenotypes, cartilage and bone, indicates that mesenchymal stem cells were present in the initial inoculum (1,2,45).

cells are counted, and $1–10 \times 10^6$ cells are placed in a small diffusion chamber (1,2,45). This chamber is of simple construction consisting of a small plastic ring onto which two Millipore filters have been glued. The filters allow body fluids (salts, nutrients, proteins, large protein complexes) to pass in and out of the chamber, but cells inside are not mixed with host cells, and tissues such as the vasculature are completely excluded. These chambers are implanted into the peritoneal cavity of an athymic (nude) mouse as an in vivo incubator, and they quickly become surrounded by host vasculature. Detailed studies have shown that the hematopoietic cells are eliminated, whereas mesenchymal cells vigorously divide and differentiate into cartilage in the middle of the chamber and bone at the filter interfaces closest to the enveloping vasculature (1,2,45). The presence of both cartilage and bone in the diffusion chamber has been compared to the presence of predominantly bone inside the highly vascularized pore regions of porous calcium phosphate ceramics loaded with marrow cells and implanted at heterotopic or orthotopic sites described above (40,41).

As a refinement of these experiments, we have been able to purify marrow mesenchymal cells by their differential adhesion to culture dishes and have successfully cultured cells through many passages (23). These cultured marrow mesenchymal cells from rat or chicken retain their capacity to differentiate into osteoblasts in ceramics through such subculturing. Of importance is the demonstrated success of isolating marrow mesenchymal cells and mitotically expanding these cells with retention of their full developmental potency to differentiate into osteoblasts or chondrocytes.

Periosteum

Another repository for mesenchymal stem cells is the periosteum, a complex layer of cells that composes the outermost layer of long bone; we have termed the periosteum the stacked cell layer in developing embryos (1,16,47,48). This layer clearly responds to injury by rapidly expanding and forming woven bone; it also has cells capable of differentiating into chondrocytes when the periosteum is transplanted into an articular cartilage defect (39). In experimentation paralleling that described above for marrow mesenchymal cells,we have been successful in culturing and passaging periosteal cells (36). In porous ceramics implanted in nude mice, these cultured periosteal cells differentiate into osteoblasts (36). When the same cell preparation is injected into a subcutaneous site in a nude mouse, the cultured periosteal cells differentiate into both bone and cartilage (36). The important point is that culture-expanded periosteal cells retain their full developmental potency and can be manipulated to form two very complex and different tissues, bone or cartilage.

THE FUTURE: (SELF-CELL THERAPY)

Several important conceptual and technical advances have converged to allow us to consider the possibility of using a patient's own mesenchymal stem cells as starting material for tissue repair protocols. Mesenchymal stem cells must exist to maintain the living organisms, just as hematopoietic stem cells must exist to support both red and white blood cell turnover. Developmental biology has taught us that differentiated cells arise in a sequence of definitive cellular and molecular transitions, a lineage, from stem cell to end phenotype. Bone, for example, turns over; new osteoblasts arise, have a defined half-life, make new bone, and then die, to be replaced by other newly differentiating end-stage osteoblasts. Such osteoblasts must arise from stem cells; thus, a living organism must have repositories of stem cells.

Therefore, we might be able to isolate such human mesenchymal stem cells and place them in cell

culture, where we could mitotically expand their numbers. Eventually, if we had enough of these cells, we could reintroduce them into the original donor in a manner that guaranteed that they would massively differentiate into a specific tissue, such as cartilage or bone, at a transplantation or repair site. Immunorejection would not be a problem because the donor and host would be one and the same.

The first experimental step to test this idea is to determine if the animal-based technology described above can be modified to be used with human material. The first attempts at this have been highly encouraging. Recently, human marrow was introduced into diffusion chambers which were placed in nude mice; both cartilage and bone were eventually observed in the chamber (2). We recently cultured human marrow and isolated mesenchymal cells that were passaged, introduced into porous ceramics, and implanted subcutaneously in nude mice. In the pore regions of these highly vascularized composites, bone clearly formed in every sample of culture-expanded, marrow-derived mesenchymal cells tested (27). These preliminary experiments provide hope that the animal-based technology developed for mesenchymal cells from marrow or periosteum will be translatable to humans.

The concept of ex vivo manipulation of cells and their reimplantation into a donor is the basis for proposing self-cell therapy as a future possibility. Massive bone regeneration to fill gaps from tumor excision, regeneration of damaged articular cartilage, and maintenance of bone formation in the elderly at risk for osteoporosis are clinical protocols that require large numbers of the appropriate reparative skeletal cells. The patient's own mesenchymal stem cells may prove to be the basis of a new, cell-based treatment plan requiring the merging of molecular biology to produce specific bioactive factors, cell biology to develop ex vivo manipulation regimens, and surgeons able to implant cells capable of repairing skeletal defects by the regeneration process.

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Articles

Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial

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See Comment

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Summary

Background **Emerging evidence suggests that stem cells and progenitor cells derived from bone marrow can be used to improve cardiac function in patients after acute myocardial infarction. In this randomised trial, we aimed to assess whether intracoronary transfer of autologous bone-marrow cells could improve global left-ventricular ejection fraction (LVEF) at 6 months' follow-up.**

Methods **After successful percutaneous coronary intervention (PCI) for acute ST-segment elevation myocardial infarction, 60 patients were randomly assigned to either a control group (n=30) that received optimum postinfarction medical treatment, or a bone-marrow-cell group (n=30) that received optimum medical treatment and intracoronary transfer of autologous bone-marrow cells 4·8 days (SD 1·3) after PCI. Primary endpoint was global left-ventricular ejection fraction (LVEF) change from baseline to 6 months' follow-up, as determined by cardiac MRI. Image analyses were done by two investigators blinded for treatment assignment. Analysis was per protocol.**

Findings **Global LVEF at baseline (determined 3·5 days [SD 1·5] after PCI) was 51·3 (9·3%) in controls and 50·0 (10·0%) in the bone-marrow cell group (p=0·59). After 6 months, mean global LVEF had increased by 0·7 percentage points in the control group and 6·7 percentage points in the bone-marrow-cell group (p=0·0026). Transfer of bone-marrow cells enhanced left-ventricular systolic function primarily in myocardial segments adjacent to the infarcted area. Cell transfer did not increase the risk of adverse clinical events, in-stent restenosis, or proarrhythmic effects.**

Interpretation **Intracoronary transfer of autologous bone-marrow-cells promotes improvement of left-ventricular systolic function in patients after acute myocardial infarction.**

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Introduction

Rapid reperfusion of the infarct-related coronary artery is of great importance in salvaging ischaemic myocardium and limiting the infarct size in patients with acute myocardial infarction. When done expeditiously and expertly, percutaneous transluminal coronary angioplasty with stent implantation is the method of choice to re-establish coronary flow.[1] Unfortunately, myocardial necrosis starts rapidly after coronary occlusion, usually before reperfusion can be achieved.[2] The loss of viable myocardium initiates a process of adverse left-ventricular remodelling, leading to chamber dilatation and contractile dysfunction in many patients.[3] In this context, much interest has followed from experimental studies showing that cardiac transfer of unfractionated bone-marrow cells, or stem cells and progenitor cells derived from bone marrow can enhance functional recovery after acute myocardial infarction.[4,5] Based on these data, stem cells and progenitor cells derived from bone marrow have been proposed for use in the repair of cardiac tissue after acute myocardial infarction in patients.[6-8]

Early clinical investigations indicate that infusion of autologous bone-marrow cells into the infarct-related coronary artery is feasible after acute myocardial infarction.[9,10] However, because these studies were not randomised trials, the efficacy of intracoronary transfer of bone-marrow cells for functional recovery after acute myocardial infarction in patients has remained uncertain. We did a randomised controlled trial to assess the effect of intracoronary transfer of autologous bone-marrow cells on left-ventricular functional recovery in patients after acute myocardial infarction and successful percutaneous coronary intervention (PCI).

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Methods

Patients

Patients were eligible if they were admitted within 5 days of the onset of symptoms of a first ST-segment elevation myocardial infarction, had undergone successful PCI with stent implantation in the infarct-related artery, and had hypokinesia or akinesia involving more than two thirds of the left-ventricular anteroseptal, lateral, and/or inferior wall, as shown by angiography done immediately after PCI. We excluded patients who had multivessel coronary artery disease, pulmonary oedema, cardiogenic shock, advanced renal or hepatic dysfunction, or documented terminal illness or cancer.

This randomised-controlled study of BOne marrOw transfer to enhance ST-elevation infarct regeneration (the BOOST trial) was approved by our local Ethics Committee. Patients provided written informed consent.

Randomisation and baseline cardiac MRI

Patients were randomly allocated in a 1:1 ratio to either the control or bone-marrow-cell groups, with use of sequentially numbered, sealed envelopes provided by IST (DM). After randomisation, all patients underwent cardiac MRI.

Harvest and transfer of bone-marrow cells

After baseline cardiac MRI, bone marrow was harvested from patients in the bone-marrow-cell group. Bone marrow was processed by 4% gelatine-polysuccinate density gradient sedimentation according to current Good Manufacturing Practice (GMP) regulations (Cytonet, Hannover, Germany), to reduce the volume of the preparation and to deplete erythrocytes and platelets. The final suspension of bone-marrow cells was washed and resuspended in saline with 10 000 U/L heparin.

We used an automated haemocytometer to measure the number of nucleated cells, packed-cell volume, and platelet count in the initial bone marrow aspirate and in the final preparation of bone-marrow cells. Nucleated cell viability was assessed by trypan blue exclusion. We measured the number of CD34+ cells with flow cytometry analysis (FACSCalibur, BD Biosciences, Heidelberg, Germany) using an antibody from Beckman Coulter (Krefeld, Germany). Haemopoietic colony-forming cell growth was measured by a methylcellulose assay (StemCell Technologies, St Katharinen, Germany).

6-8 h after bone-marrow harvest, the final preparation of bone-marrow cells was infused into the infarct-related artery via the central lumen of an over-the-wire balloon catheter (Concerto, Occam International, Eindhoven, Netherlands). To allow bone-marrow cells maximum contact time with the microcirculation of the infarct-related artery, the balloon was inflated inside the stent to transiently interrupt antegrade blood flow during infusions. The entire bone-marrow-cell preparation was infused during four to five coronary occlusions, each lasting 2·5-4 min. Between occlusions, the coronary artery was reperfused for 3 min.

Follow-up

All patients were treated with aspirin (300 mg daily for 4 weeks after PCI, then 100 mg daily), clopidogrel (300 mg loading dose, then 75 mg daily for at least 4 weeks after PCI), an angiotensin-converting enzyme (ACE) inhibitor or angiotensin-receptor blocker, a ßblocker, and a statin (if LDL

cholesterol concentrations were above 2·6 mmol/L), unless these agents were contraindicated. At both 6 weeks and 3 months after discharge, patients had follow-up examinations to assess their clinical status and to review their current medication. Where necessary, dosages of angiotensin-converting enzyme inhibitors (ACE-inhibitors), angiotensin-receptor blockers, ßblockers, and statins were adjusted in accordance with current practice guidelines.[11,12] 6 months after discharge, cardiac MRI was repeated in all patients. In addition, patients were scheduled to undergo coronary angiography to assess the degree of restenosis in the stented segment of the infarct-related artery. Restenosis was quantified with a computer-based system (CMS, Medical Imaging Systems, Leiden, Netherlands) by an investigator unaware of treatment assignment (AM).

To assess whether intracoronary bone-marrow-cell transfer was associated with proarrhythmic effects, we obtained 24 h Holter recordings from all patients before hospital discharge, and at 6 weeks', 3 months', and 6 months' follow-up. From these recordings, the mean number of premature ventricular complexes per h was calculated. We also recorded the number of non-sustained and sustained ventricular tachycardias per recording. In addition, patients were scheduled to undergo programmed ventricular stimulation at 6 months' follow-up. Ventricular stimulation was done at the right-ventricular apex and the right-ventricular outflow tract with single, double, and triple extra stimuli at twice the diastolic threshold and basic cycle lengths of 500 ms and 400 ms.

Cardiac MRI

Cardiac MRI was done with the patient in supine position in a 1.5-T scanner (CV/i, General Electric, Munich, Germany) using electrocardiogram (ECG) gating and a four-element phased array receiver coil. To measure left-ventricular volumes, we used repeated breath-hold fast gradient echo sequences in a steady state (FIESTA, General Electric). Sequence parameters were as follows: TR/TE 3·8/1·6 ms, 40° flip angle, 224x224 matrix, field of view 36-38 cm, in-plane resolution 1·6x1·6-1·7x1·7 mm, 38-40 phases per RR-interval, 10 mm slice thickness. An end-diastolic, horizontal long-axis plane of the left ventricle at end-expiration provided the reference image on which a stack of contiguous short-axis slices was positioned to cover the entire left ventricle.

Contrast-enhanced MRI was used to assess myocardial injury after acute myocardial infarction.[13] A breath-hold k-space segmented T1 weighted inversion recovery gradient echo sequence was used to cover the entire left ventricle with 7-8 mm short-axis slices as described above (TR/TE 7·1/3·1 ms, 256x192 matrix, field of view 36-38 cm, in-plane resolution 1·4x1·9-1·5x2·0 mm). Inversion time (200-220 ms) was individually adapted to null the signal of the myocardium. End-diastolic images were obtained starting 15 min after an intravenous bolus injection of 0·15 mmol/kg gadobutrol, a gadolinium-based extracellular contrast agent (Schering, Berlin, Germany).

All image analyses were done by two investigators who were unaware of treatment assignment (CB and SF), using the MASS 4.0.1 software (Medical Imaging Systems). Endocardial and epicardial borders were traced in all end-diastolic and end-systolic short-axis slices to determine left-ventricular end-diastolic volumes (LVEDV) and end-systolic volumes (LVESV) for global and regional calculation of left-ventricular ejection fraction (LVEF), and left-ventricular mass. For assessment of infarct volumes, late contrast enhancement was quantified. LVEDV index, LVESV index, and left-ventricular-mass index were calculated by dividing LVEDV, LVESV, and left-ventricular mass by body surface area. Regional LVEF was derived by calculating LVEF only in slices showing late contrast enhancement at

baseline. Regional left-ventricular function was assessed by determining systolic wall motion in the infarct region and border zone. Systolic-wall motion was defined as the radial displacement of the endocardial contour at systole. Myocardial segments showing late contrast enhancement at baseline were defined as the infarct region. Segments adjacent to the infarct region were defined as the border zone.

Statistical analysis

Primary endpoint was the change from baseline in global LVEF at 6 months' follow-up. Secondary endpoints were changes in LVEDV index, LVESV index, left-ventricular-mass index, and late contrast enhancement. We calculated that we would need 30 patients in each group to achieve a power of at least 80% to detect a difference in global LVEF change of 5 percentage points between study groups, with a two-sided significance level of $p<0·05$, and a common standard deviation of 6·5 percentage points for the global LVEF change from baseline to 6 months' follow-up. We used ANCOVA to compare global LVEF changes in the two study groups, with bone-marrow-cell treatment as the main factor and LVEF at baseline as a covariate. To estimate the treatment effect, differences in least-squares means and corresponding 95% CI were calculated based on the ANCOVA model. We analysed secondary endpoints using the same methods. The consistency of the treatment effect on global LVEF change was assessed across several subgroups. All statistical tests were two-sided with a significance level of $p<0·05$.

Homogeneity of treatment groups at baseline was assessed using Student's *t* test for continuous variables showing no marked deviations from the normal distribution. For other continuous variables or ordinal baseline data, the Wilcoxon rank-sum test was used. Categorical baseline data were investigated using χ^2 tests. The relation between the number of nucleated cells, CD34+ cells, and haemopoietic colony-forming cells infused into the infarct-related coronary artery and subsequent global LVEF changes were assessed with Pearson's correlation coefficient. Subgroup analyses were not prespecified but were exploratory in nature. All subgroup analyses are reported.

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Results

Between January, 2002, and May, 2003, 78 patients were informed about the trial. 65 patients were randomly allocated to treatment. After randomisation, five patients were withdrawn because they could not undergo cardiac MRI, either because of claustrophobia or severe obesity. The final cohort included 30 controls and 30 patients in the bone-marrow-cell group (figure 1). Table 1 shows patients' baseline characteristics. All patients received optimum postinfarction medical treatment (table 1).

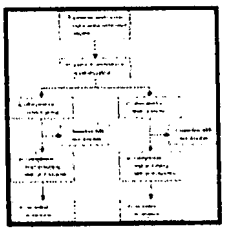

***Figure1:*Trial profile**

BMC=bone-marrow cell.

	Control group (n=30)	BMC group (n=30)	p
Age (years)	59·2 (13·5)	53·4 (14·8)	0·11
Men	22 (73%)	20 (67%)	0·57
Body-mass index (kg/m^2)	26·2 (4·2)	25·8 (3·0)	0·67
Diabetes mellitus	3 (10%)	3 (10%)	1·0
Hyperlipidaemia*	7	9	0·56
Hypertension	13 (43%)	9 (30%)	0·28
Current cigarette use (number of patients)	17 (57%)	18 (60%)	0·79
Median time from symptom onset to PCI (h) (range)	8·0 (3-120)	9·8 (2-22)	0·92
Killip class			
1	25 (83%)	23 (77%)	0·51
2	5 (17%)	7 (23%)	
3 or 4	0	0	
Infarct-related artery			
Right coronary artery	7 (23%)	7 (23%)	1·0
Left coronary artery	23 (77%)	23 (77%)	
TIMI flow grade			
before PCI:			0·73
Grade 0 or I	16 (53%)	13 (43%)	
Grade II	13 (43%)	16 (53%)	
Grade III	1 (3%)	1 (3%)	
after PCI:			0·75
Grade 0 or I	0	0	
Grade II	7 (23%)	6 (20%)	
Grade III	23 (77%)	24 (80%)	
Maximum serum creatine kinase concentration (U/L)	2844 (1161)	2968 (1867)	0·77
Maximum serum creatine kinase MB concentration (U/L)	156 (51)	175 (123)	0·46
Maximum serum troponin T concentration (µg/L)	7·4 (4·4)	7·4 (5·5)	0·99
Periprocedural therapy			
Thrombolytic therapy before PCI	10 (33%)	14 (47%)	0·29
Platelet glycoprotein IIb/IIIa inhibitors	14 (47%)	14 (47%)	1·0
Median number of stents (range)	1 (1-5)	1 (1-2)	0·40
Size of stent (mm)	3·3 (0·4)	3·3 (0·4)	1·0
Length of stent (mm)	17·5 (9·6)	17·6 (6·4)	0·97
Lesion characteristics			0·71
Type A	8 (27%)	6 (20%)	
Type B	16 (53%)	19 (64%)	
Type C	6 (20%)	5 (3%)	
Medication at primary discharge:			
Aspirin† and clopidogrel	29 (97%)	30 (100%)	
ACE-inhibitors or angiotensin-	30 (100%)	30 (100%)	

receptor blockers		
ß blockers	30 (100%)	29 (97%)
Statins	29 (97%)	30 (100%)
at 6 months' follow-up:		
Aspirin†	27 (97%)	29 (97%)
ACE-inhibitors or angiotensin-receptor blockers	30 (100%)	30 (100%)
ß blockers	30 (100%)	29 (97%)
Statins	28 (93%)	28 (93%)

BMC=bone-marrow cell. ACE=angiotensin-converting enzyme. Data are means (SD) or n (%) unless otherwise stated. *Serum cholesterol >5·2 mmol/L. †Patients not receiving aspirin were treated with phenprocoumon.

Table 1: **Patients' characteristics**

Mean time from PCI to baseline cardiac MRI was 3·5 days (SD 1·5). Mean time from PCI to bone-marrow harvest was 4·8 days (1·3). Time from symptom onset to harvest of bone-marrow cells was 5·7 days (1·2). On average, 128 mL (33) of bone marrow was aspirated from the posterior iliac crest during a brief general anaesthesia with midazolam and etomidate. No bleeding complications at the harvest site were noted.

During preparation of bone-marrow cells, the sedimentation process reduced the volume of bone-marrow cells to a mean of 26 mL (SD 4) and recovered 75% (12) of nucleated cells from the initial bone-marrow aspirate. The final preparation of bone-marrow cells contained $24{\cdot}6x10^8$ (SD $9{\cdot}4x10^8$) nucleated cells (viability 99% [2]), $9{\cdot}5x10^6$ ($6{\cdot}3x10^6$) CD34+ cells, and $3{\cdot}6*10^6$ ($3{\cdot}4x10^6$) haemopoietic colony-forming cells. The packed cell volume of the final bone-marrow-cell preparation was 31% (11), and the platelet count was $182x10^6$ ($93x10^6$) per mL .

Changes of LVEDV index, LVESV index, left-ventricular-mass index, and late-contrast enhancement from baseline to 6 months' follow-up did not differ significantly between the control and bone-marrow-cell groups (table 2). The increase in LVEDV index at 6 months was slightly higher in the bone-marrow-cell group, whereas LVESV index tended to decrease more in the bone-marrow-cell group (table 2). 6 months after randomisation, global LVEF increased significantly in the bone-marrow-cell group compared with controls (p=0·0026) (table 2 and figure 2). The effects of bone-marrow-cell transfer on global LVEF change at 6 months' follow-up were consistent in all investigated subgroups (figure 3). The improvement in global LVEF after 6 months' follow-up was not correlated with the number of nucleated cells (r= -0·11, p=0·57), CD34+ cells (r=0·13, p=0·48), or haemopoietic colony-forming cells (r= -0·14, p=0·46) infused into the infarct-related coronary artery.

	Baseline		**6 months**		**Change**		**BMC treatment effect***	**p**
	Controls	BMC group	Controls	BMC group	Controls	BMC group		
LVEDV index (mL/m^2)	81·4 (16·9)	84·2 (17·2)	84·9 (21·9)	91·7 (26·0)	3·4 (11·1)	7·6 (20·0)	4·0 (-4·4 to 12·5)	0·32
LVESV index	40·6 (16·9)	43·0 (14·7)	42·6 (23·5)	42·4 (23·9)	2·0 (11·1)	-0·6 (14·9)	-3·2 (-9·7 to 3·3)	0·33

(mL/m^2)								
Global LVEF (%)	51·3 (9·3)	50·0 (10·0)	52·0 (12·4)	56·7 (12·5)	0·7 (8·1)	6·7 (6·5)	6·0 (2·2 to 9·9)	0·0026
LVM index (g/m^2)	78·2 (18·3)	82·7 (18·7)	71·7 (14·2)	71·9 (14·6)	-6·5 (12·8)	-10·8 (10·6)	-2·5 (-7·3 to 2·3)	0·30
LE (mL)	30·3 (17·4)	33·0 (21·1)	19·8 (9·8)	18·9 (12·2)	-10·5 (10·6)	-14·1 (13·0)	-2·2 (-5·4 to 1·0)	0·18

BMC=bone-marrow cell. Data are mean (SD) unless otherwise stated. *Treatment effects expressed as differences in least-squares means (ANCOVA model) with 95% CI. LVM=left ventricular mass. LE=late contrast enhancement. There were no differences between groups at baseline.

Table 2: **Left ventricular volume and mass indices, global LVEF, and late enhancement as determined by contrast-enhanced MRI at baseline and 6 months' follow-up**

Figure2: **Global LVEF at baseline and 6months' follow-up**

*p=0·0026 for difference between groups. Small dots show data for individual patients; large dots show mean values. Vertical bars show SD.

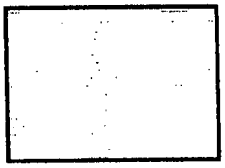

Figure3: **Subgroup analyses of global LVEF changes from baseline at 6months' follow-up**

LE=late contrast enhancement. BMC=bone-marrow cell. *Median values of the whole study population were used to create subgroups of equal size. ŶCardiovascular risk factors were diabetes, total cholesterol concentration greater than 5·2 mmol/L , hypertension, or current smoking. Oval dots show differences of least-squares means between groups; horizontal bars show 95% CI.

Compared with the control group, patients in the bone-marrow-cell group had increased regional LVEF (p=0·04) and systolic wall motion in the border zone (p=0·03) at 6 months. By contrast, systolic wall motion in the infarct region was not significantly enhanced by transfer of bone-marrow-cells (table 3). Representative colour-coded images showing the effects of bone-marrow-cell transfer on left-ventricular function are shown in figure 4.

	Baseline		**6 months**		**Change**		**BMC treatment effect†**	**p**
	Controls	BMC group	Controls	BMC group	Controls	BMC group		
Regional LVEF	47·8 (9·7)	46·3 (10·6)	48·9 (15·2)	53·0 (15·5)	1·1 (11·8)	6·7 (9·5)	5·7 (0·2 to 11·3)	0·04

(%)							
Systolic wall motion (mm), infarct region	3·9 (1·8)	4·4 (1·9)	4·9 (2·9)	5·9 (2·5)	1·0 (2·5)	1·5 (2·1)	0·6 (-0·6 to 1·8)	0·32
Systolic wall motion (mm), border zone	6·8 (1·6)	7·0 (1·7)	6·8 (2·1)	8·0 (2·1)	-0·1 (2·2)	1·0 (1·9)	1·1 (0·1 to 2·1)	0·03

BMC=bone-marrow cell. Data are mean (SD). Treatment effects are expressed as differences in least-squares means (ANCOVA model) and 95% CI· There were no differences between groups at baseline·

Table 3: **Regional LVEF and systolic wall motion as determined by contrast-enhanced MRI at baseline and 6 months' follow-up**

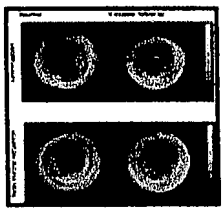

Figure4: **Representative colour-coded images showing systolic wall motion at baseline and 6months' follow-up in two patients**

Both patients had had an anterior acute myocardial infarction. Bright colours indicate good systolic wall motion, whereas dark colours indicate poor wall motion (expressed in mm). Note improved functional recovery in the patient treated with bone-marrow-cells.

No patient died or was lost to follow-up. There were no increases in troponin T concentrations in serum in any of the patients 24 h after intracoronary transfer of bone-marrow cells, indicating that the procedure did not inflict additional ischaemic damage to the myocardium. In 6 months of follow-up, three controls and one patient from the bone-marrow-cell group needed at least one hospital admission for worsening heart failure. One person from the bone-marrow-cell group developed a non ST-segment elevation myocardial infarction in the left circumflex territory 4 months after transfer of bone-marrow-cells into the left anterior descending coronary artery. This patient underwent PCI of the left circumflex coronary artery and completed the study.

There were no differences between the control and bone-marrow-cell groups with respect to the number of premature ventricular complexes per h and the occurrence of non-sustained or sustained ventricular tachycardias by Holter monitoring at 6 weeks', 3 months', and 6 months' follow-up. 28 (93%) controls and 27 (90%) patients who had bone-marrow-cell transfer agreed to undergo an electrophysiological study at 6 months' follow-up. A non-sustained ventricular tachycardia was inducible in one control patient and in one bone-marrow-cell transfer patient. Ventricular fibrillation was inducible in one control patient. In 29 (97%) controls and 28 (93%) patients who had bone-marrow-cell transfer, coronary angiograms were obtained at 6 months' follow-up. Mean in-stent restenosis in the infarct-related artery, expressed as a percentage of luminal diameter, was 32% (SD 20) in the control group and 33% (23) in the bone-marrow-cell group (p=0·88). Four

patients from the control group and seven from the bone-marrow-cell group presented with an in-stent restenosis of at least 50% (p=0·28). One patient from the control group developed total in-stent occlusion.

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Discussion

Our randomised controlled clinical trial addresses the effect of autologous bone-marrow-cell therapy on left-ventricular functional recovery after acute ST-segment elevation myocardial infarction. We have shown that infusion of autologous bone-marrow-cells into the infarct-related coronary artery during the early postinfarction period (4-8 days after symptom onset) improves recovery of global LVEF after 6 months.

In view of the size of our trial, subgroup analyses must be considered with caution. With this caveat in mind, it is noteworthy that the effects of bone-marrow-cell transfer on global LVEF change were consistent across all investigated subgroups. The effects of cell transfer were over and above benefits associated with established strategies to promote functional recovery after acute myocardial infarction, such as PCI with stent implantation, and postinfarction pharmacotherapy with ACE-inhibitors, angiotensin-receptor blockers and ßblockers.[11,12]

Global LVEF at baseline was 51% (SD 10) in our patient cohort, which is consistent with previous MRI studies in patients after myocardial infarction.[14,15] In healthy adults, normal LVEF values of 67% (5) have been shown with MRI.[16] Therefore, patients enrolled in our study had substantial functional impairment. Global LVEF increased by only 0·7 percentage points after 6 months' in the control group, emphasising the need for additional therapeutic strategies to enhance functional recovery in patients with acute myocardial infarction. Since 40% of patients had been transferred for rescue PCI from outside hospitals, the average time from symptom onset to PCI was quite long in our trial (median 8·5 h). Previous studies have shown that greater LVEF improvement (up to 4 percentage points) can be achieved when coronary patency is re-established within 4 h of symptom onset.[17,18] Of note, however, is that in these studies baseline LVEF was measured within 24 h of PCI.[17,18] By contrast, we assessed baseline LVEF 3·5 days (SD 1·5) after PCI, at a time when left-ventricular function is likely to have partly recovered from postischaemic myocardial dysfunction (ie, stunning).[19] Similar to the results obtained in our control group, two MRI studies that used serial LVEF measurements in patients with reperfused myocardium after acute myocardial infarction have reported no significant improvement in LVEF from a baseline investigation at day 5-7, to follow-up at 3-6 months.[14,15]

Improvement of global LVEF in the treatment group was due mostly to improved regional systolic wall motion in the infarct border zone. Left-ventricular end-diastolic volumes did not decrease, indicating that transfer of bone-marrow-cells did not improve left-ventricular remodelling at 6 months. Longer follow-up of our patients is required (and will be done) to assess the impact of bone-marrow-cell transfer on long-term left-ventricular structural adaptation after acute myocardial infarction.

Because of ethical considerations, we decided not to do bone-marrow aspiration and a sham left-heart catheterisation in patients randomised to the control group. Importantly, however, all MRI data were analysed by two investigators who were not aware of treatment assignments.

Our study was not designed to assess underlying mechanisms of treatment with bone-marrow-cells that promote functional recovery after acute myocardial infarction. Apparently, transdifferentiation of bone-marrow-derived haemopoietic stem cells to cardiomyocytes cannot account for the beneficial effects.[20,21] Instead, recent papers have highlighted the potential of bone-marrow cells to promote paracrine effects in ischaemic tissues (eg, secretion of angiogenic factors), and suggest that paracrine signalling, rather than cell incorporation, promotes functional recovery.[5,22-25]

Our experience suggests that intracoronary bone-marrow-cell transfer is safe; specifically, there was no evidence for an increased rate of in-stent restenosis or proarrhythmic effects. It should be noted that high rates of in-stent restenosis have been reported after intracoronary transfer of granulocyte colony-stimulating-factor mobilised peripheral-blood mononuclear cells.[26] Importantly, granulocyte colony-stimulating factor, which may promote in-stent restenosis by enhancing neutrophil recruitment at sites of tissue injury,[27] was not used in our study. Intracoronary injection of bone marrow-derived mesenchymal stromal cells has been shown to cause microinfarctions in dogs.[28] It should be noted that nucleated bone-marrow cells are significantly smaller than expanded mesenchymal stromal cells ex vivo,[28] which may explain why we, and others,[10] did not observe infarctions (ie, increases in concentrations of troponin T in serum) after intracoronary transfer of bone-marrow cells.

Our results lend support to the concept that autologous bone-marrow cells can be used to enhance left-ventricular functional recovery in patients after acute myocardial infarction. Larger trials are needed to address the effect of bone-marrow cell transfer on clinical endpoints such as the incidence of heart failure and survival.

Contributors

K C Wollert contributed to study design, enrolment, and clinical follow-up of patients, aspiration and intracoronary transfer of bone marrow, and the writing of the manuscript. G P Meyer contributed to study design, enrolment of patients, MRI data acquisition, and intracoronary BMC transfer. J Lotz contributed to MRI data acquisition. C Breidenbach and S Fichtner analysed MRI data. S Ringes-Lichtenberg contributed to enrolment and clinical follow-up of patients. T Korte did electrophysiological studies. B Hornig did intracoronary transfer of bone-marrow cells. P Lippolt and D Messinger did statistical analyses. L Arseniev did bone-marrow-cell sedimentations. B Hertenstein and A Ganser contributed to study design and did bone-marrow aspirations. H Drexler contributed to study design and the writing of the manuscript.

Conflict of interest statement

L Arseniev is business unit leader of Cytonet Hannover, the company that did the bone-marrow-cell sedimentations during the trial. L Arseniev has not been involved in any way in MRI data collection or data analysis in this trial.

Acknowledgments

Kai C Wollert and Gerd P Meyer contributed equally to this work. We thank Alix Menke and Dieter Fischer for analysing the coronary angiograms; our colleagues and nurses at the Departments of Cardiology and Angiology and Diagnostic Radiology, and at Cytonet Hannover for their support during the trial. The trial was supported by internal funding from the Department of

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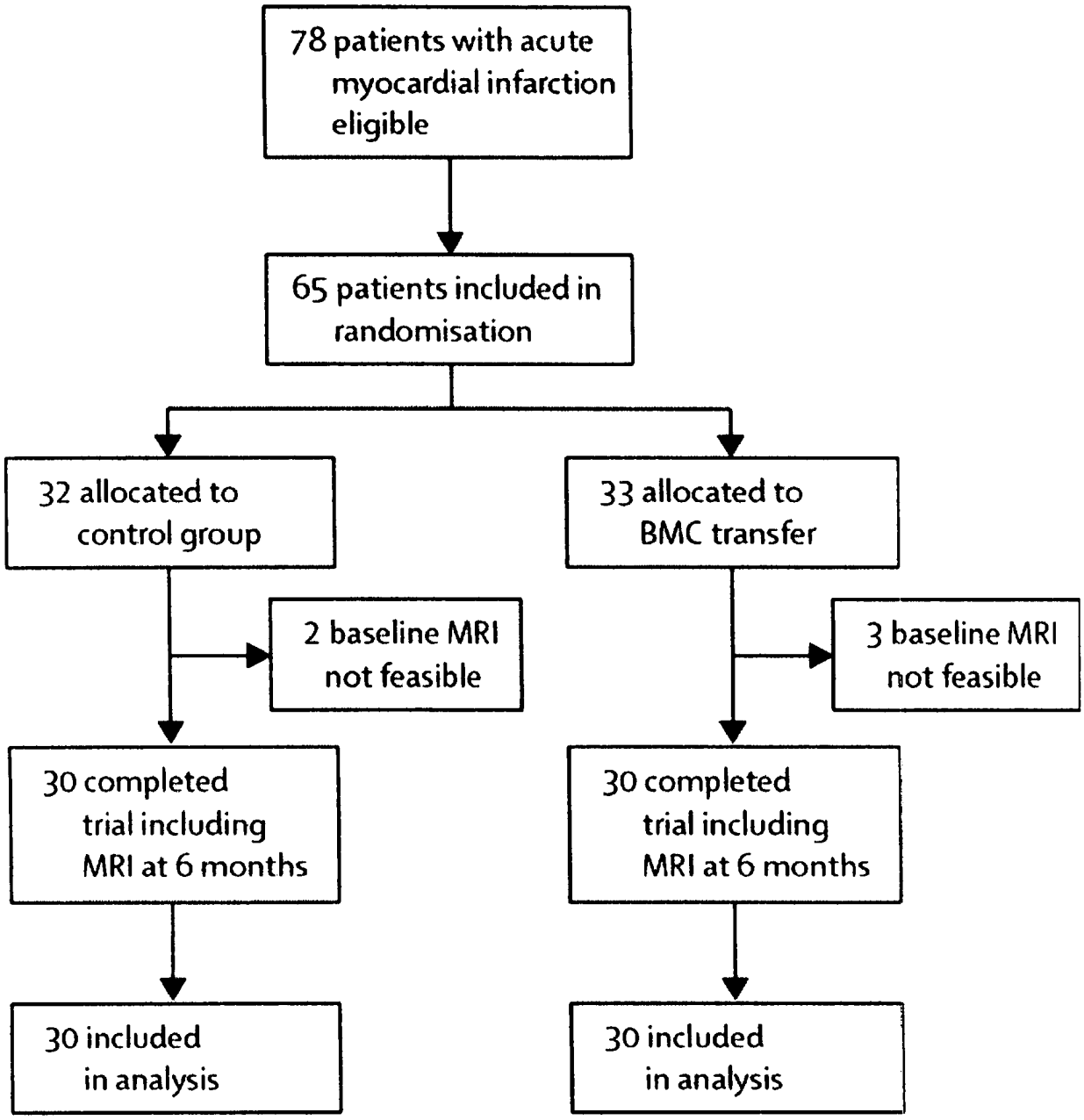

Figure1:Trial profile

Cardiology.

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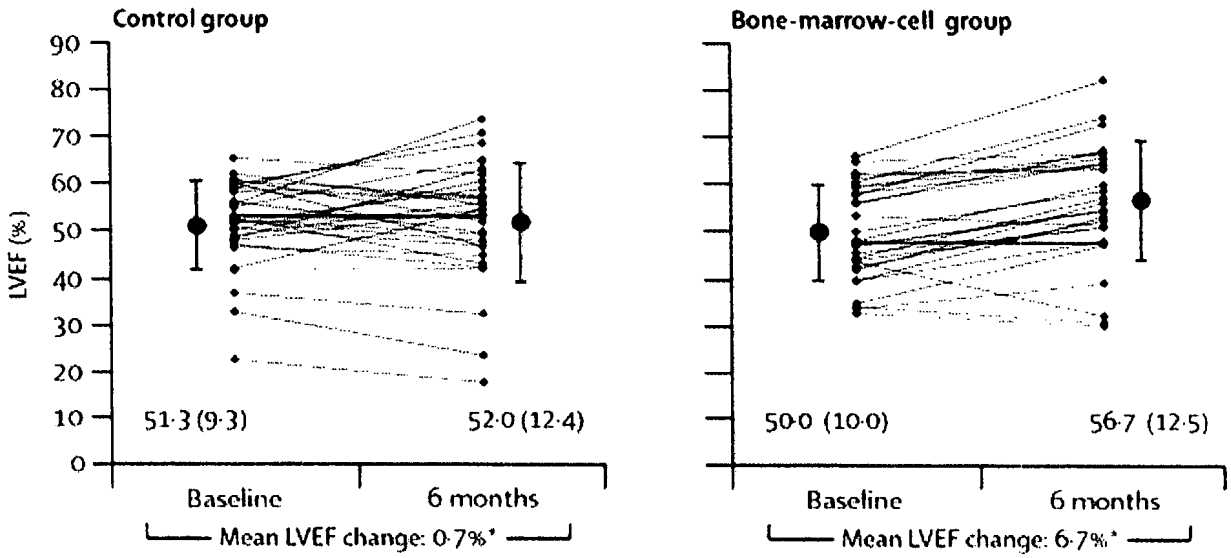

Figure2:Global LVEF at baseline and 6months' follow-up

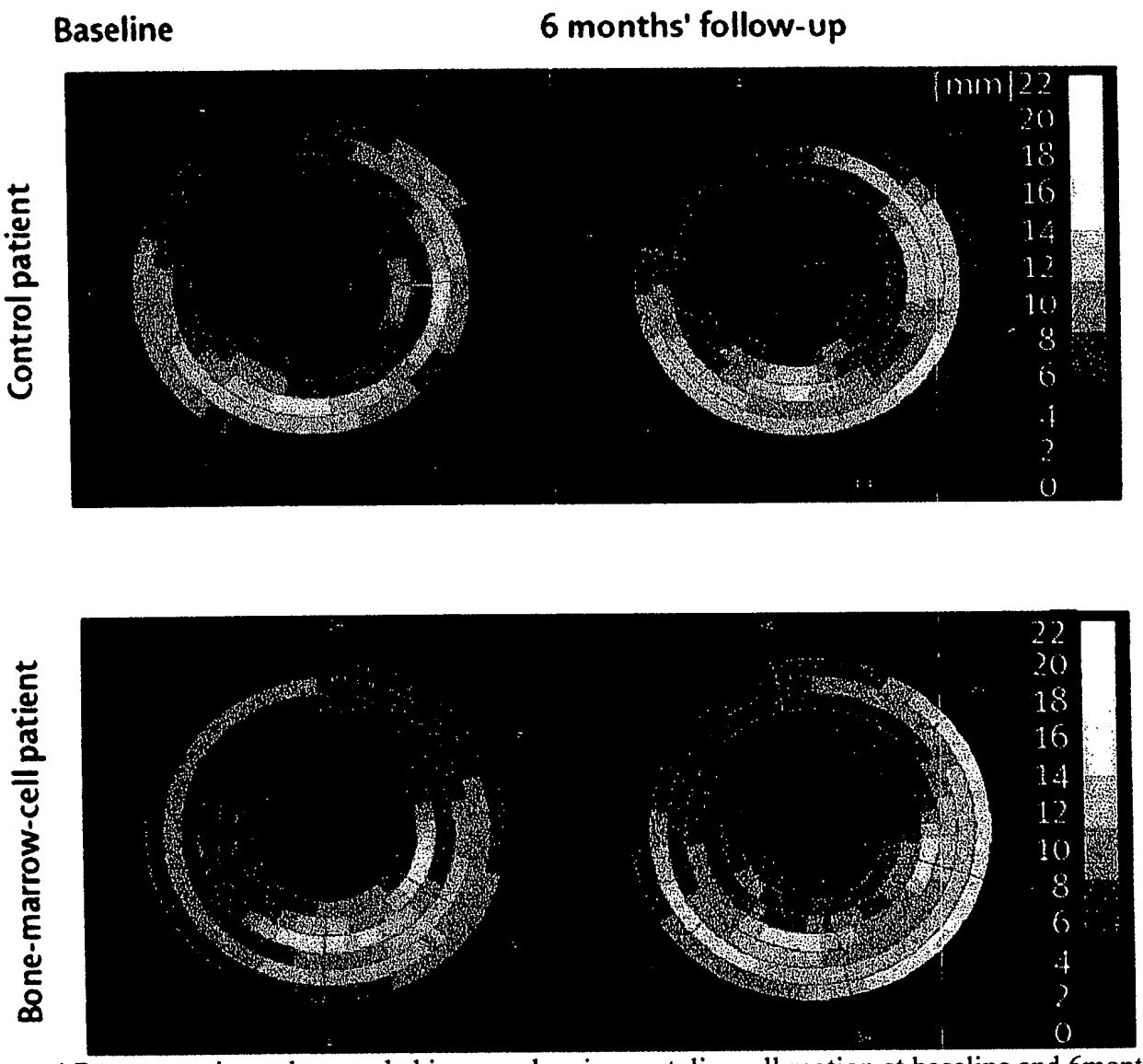

Figure4:Representative colour-coded images showing systolic wall motion at baseline and 6months' follow-up in two patients

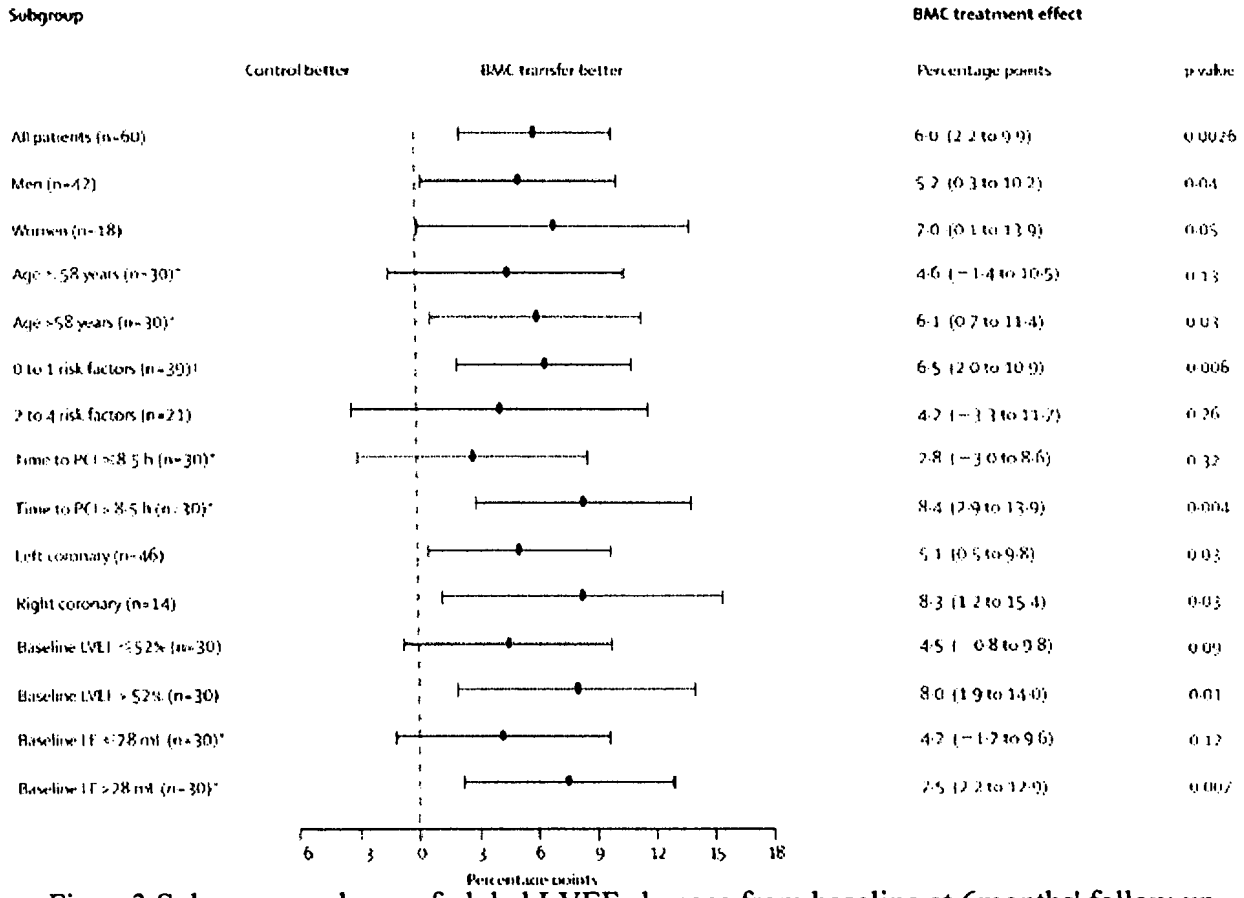

Figure3:Subgroup analyses of global LVEF changes from baseline at 6months' follow-up

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CERTIFICATE OF MAILING

I hereby certify that the attached DECLARATION OF RICHARD HEUSER, M.D. was delivered to the Assistant Commissioner for Patents by the undersigned from Arrow Intellectual Property Services, 2001, Jefferson Davis Highway, Suite 602, Arlington, Virginia 22202, by hand carrying said DECLARATION to Art Unit 1646, Crystal Plaza 1, Tenth Floor, Attention: Examiner Elizabeth C. Kemmerer this 17th day of June, 2003.

Dated: June 17, 2003

Ann Rutledge

Printed Name: Ann Rutledge

ARROW INTELLECTUAL PROPERTY SERVICE

Docket No: 1000-10-C01

Serial No.: 09/836,750

Filed/Received: 04/17/01

Due Date: - - -

The Patent Office acknowledges, and has stamped hereon, the date of receipt of the items check below:

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia
SERIAL NO.: 09/836,750
FILED: April 17, 2001
FOR: METHOD AND APPARATUS FOR INSTALLATION OF DENTAL IMPLANT

EXAMINER: E.C. Kemmerer, Ph.D.

GROUP ART UNIT: 1646

DECLARATION OF RICHARD HEUSER, M.D.

I, Richard Heuser, declare as follows:

1. I have offices at 525 North 18th Street, Suite 504, Phoenix, Arizona 85006.

2. My Curriculum Vitae is attached hereto as Exhibit A.

3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. A copy of such disclosures is attached hereto as Exhibit B.

4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue and, more specifically, to a method of using a growth factor for growing muscle in a human heart.

5. I am aware of and have considered the definition of *growth factor* in the specification of the above-referenced patent application at page 20, line 10 through page 21, line 15. Such definition is set forth in Exhibit C along with a definition from the medical dictionary, MEDLINE plus: Merriam-Webster Medical Dictionary. A service of the U.S. NATIONAL LIBRARY OF MEDICINE and the NATIONAL INSTITUTES OF HEALTH. I find that the dictionary definition is consistent with that contained at page 20, line 10 through page 21, line 15 of the above-referenced patent application. I believe that both definitions are appropriate for use in the field of tissue growth and would be understood by one skilled in the medical arts. Accordingly, I am adopting and utilizing the definition contained in the patent application throughout this declaration.

6. I have read and understood the claims set forth in Exhibit D and have been informed that such claims will be presented to the Patent and Trademark Office in the near future.

7. The materials included in attached Exhibit E illustrate that placement of a growth factor in a human patient causes muscle growth in a heart. These materials report work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

8. Based upon above Paragraphs 3-7, it is my opinion that introducing a growth factor into a human patient will predictably cause new muscle growth in the heart of the patient.

9. Based upon above Paragraphs 3-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit D without need for resorting to undue experimentation.

10. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 6/5/13

Richard Heuser

EXHIBIT A

CURRICULUM VITAE

Curriculum Vitae
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1969 - 1972	University of Wisconsin Honors in Chemistry Phi Beta Kappa Evan Helfaer Scholarship in Chemistry
1972 - 1976	University of Wisconsin School of Medicine Graduation with Honors – May 1976 Alpha Omega Alpha Evan Helfaer Scholarship in Medicine

POST GRADUATE TRAINING:

1976 - 1977	Internship in Medicine The Johns Hopkins Hospital Baltimore, Maryland
1977 - 1979	Residency in Medicine The Johns Hopkins Hospital Baltimore, Maryland
1979 - 1981	Fellowship in Cardiology The Johns Hopkins Hospital Baltimore, Maryland

LICENSURE:

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State of New Mexico, License #83-220

EMPLOYMENT:

December 2002 - Present	Director of Cardiovascular Research St. Joseph's Hospital and Medical Center Phoenix, Arizona
April 2001 - Present	Cardiac Cath Lab Director St. Luke's Medical Center, Phoenix, Arizona
June 2000 - Present	Medical Director Discovery Alliance, Phoenix, Arizona
1998 - June 2000	Director Phoenix Research Center, Phoenix, Arizona

April 1997 - Present	Medical Director Phoenix Heart Center, Phoenix, Arizona
December 1999 - Present	Director of Research St. Luke's Medical Center, Phoenix, Arizona
April 1997 - December 1999	Director of Research and Education Phoenix Regional Medical Center, Phoenix, Arizona
April 1990 - April 1997	Director of Research and Education Arizona Heart Institute, Phoenix, Arizona
July 1983 - April 1990	Private Practice New Mexico Heart Clinic, Albuquerque, New Mexico
July 1982 - June 1983	Private Practice Houston Cardiovascular Associates, Houston, Texas
June 1981 - July 1982	Instructor in Medicine, Cardiology The Johns Hopkins Hospital, Baltimore, Maryland

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July 1982 - June 1983	Instructor in Medicine, Cardiology Baylor College of Medicine, Houston, Texas
July 1983 - February 1990	Director, Interventional Cardiology New Mexico Heart Clinic, Albuquerque, New Mexico
April 1984 - June 1986	Clinical Assistant Professor of Medicine University of New Mexico, Albuquerque, New Mexico
	Director, Medical Residency Program New Mexico Heart Clinic, Albuquerque, New Mexico
June 1986 - April 1990	Clinical Associate Professor of Medicine University of New Mexico, Albuquerque, New Mexico
May 1996 - April 1997	Director, Interventional Cardiology Arizona Heart Institute Foundation, Phoenix, Arizona
Sept 1995 - December 1999	Medical Director - Cardiac Catheterization Laboratory Phoenix Regional Medical Center, Phoenix, Arizona
December 1990 - Present	Clinical Associate Professor of Medicine University of Louisville, Louisville, Kentucky
April 1990 - April 1997	Director of Research and Education Arizona Heart Institute Foundation, Phoenix, Arizona

April 1997 - December 1999 Director of Research and Education
Phoenix Regional Medical Center, Phoenix, Arizona

BOARD MEMBERSHIPS:

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American Board of Cardiovascular Diseases, Diplomat
American Board of Interventional Cardiovascular Diseases, Diplomat

PROFESSIONAL MEMBERSHIPS:

Fellow, American College of Angiology
Fellow, American College of Cardiology
Fellow, American College of Physicians
Fellow, of the American Heart Association
Fellow, American Society of Cardiovascular Interventions
Fellow, International Society of Cardiovascular Interventions
Fellow, Society for Cardiac Angiography and Interventions
Member, American Association for the Advancement of Science
Member, American Heart Association
Member, American Medical Association
Member, Houston Cardiology Society
Member, Houston Society of Internal Medicine
Member, International Andreas Grüntzig Society
Member, International Network of Interventional Cardiology
Member, International Society for Carotid Artery Therapy
Member, International Society for Minimally Invasive Cardiac Surgery
Member, New Mexico Medical Society
Member, Harris County Medical Society
Member, Texas Medical Association
Member, National Register's Who's Who in Executives and Professionals
Member, Who's Who in Medicine and Healthcare 2002-2003

CLINICAL ADVISORY BOARDS:

Advanced Cardiovascular Systems
USCI
Mansfield Scientific Interventional Board
Medtronic Interventional Vascular
Scientific Advisory Board of International Society of Heart Failure

EDITORIAL BOARDS:

Catheterization and Cardiovascular Diagnosis
Journal of Endovascular Surgery
Cardiovascular Research Foundation/Society or Cardiac Angiography and Interventions
Abstract Grader TCT

DATA SAFETY BOARDS:

- ICEM Data Safety Monitoring Board

- Abbott Laboratories Data Safety Monitoring Board for Drug Coated Stent Program, PREFER, A Perspective STUDY to Evaluate the Safety and Efficacy of the ABT-578 coated BiodivYsio® Stent for the Reduction of Resetnosis

CONSULTANT TO:

Editors of the *Annals of Internal Medicine*
Editors of *Catheterization and Cardiovascular Diagnosis*
Editors of *Circulation*
Editors of the *Journal of Invasive Cardiology*
Editors of the *American Journal of Cardiology*
Editors of *Web M.D.*
Annual Scientific Session Program Committee of the American College of Cardiology
Annual Scientific Session Program Committee of the American College of Cardiology
Abstract Advisor for Angioplasty; Stents
Annual International Symposium of Transcatheter Cardiovascular Therapeutics
Abstract Grader

DEVICE RESEARCH:

Sub-Investigator ACS Multi-Link Stent Trial Principal Investigator - ACS RX
Principal Investigator ACT-One Trial Principal Investigator - Angio-Seal Trial
Principal Investigator Balloon Expandable Intraluminal Stent for Subtotally Occluded Iliac Arteries
Principal Investigator Bard® Memotherm Carotid Stent Study
Principal Investigator BARRICADE Trial - The Barrier Approach to Restenosis: Restrict Intima and Curtail Adverse Events (JOMED JOSTENT)
Principal Investigator BEST Trial
Principal Investigator BetaCath System Trial
Principal Investigator Boehringer Ingelheim Pharmaceutics Protocol Comparing Micardis and COZAAR
Principal Investigator CABERNET Clinical Trial - Carotid Artery Revascularization using the Boston Scientific EPI FiltreWire EX™ and the EndoTex™ NexStent™
Principal Investigator CADILLAC Trial
Principal Investigator CAPRICORN Trial
Principal Investigator CAPTIVE - Cardioshield Application Protects During Transluminal Intervention of Vein Grafts by Reducing Emboli
Principal Investigator CARDIOMETRICS
Principal Investigator Carotid Wallstent Trial
Principal Investigator CAVEAT II Trial
Principal Investigator Clinical Investigation of the Magnum Wire vs. Standard Guide Wires during Total Occlusion Angioplasty
Principal Investigator Cook GR II Trial
Principal Investigator CORDIS Nitinol Carotid Stent And Delivery System for the Treatment of Obstructive Carotid Artery Disease
Principal Investigator Cordis Carotid Randomized Sapphire
Principal Investigator Cordis Bilateral AAA Device & Delivery System
Principal Investigator (CATS) Safe-Steer™ Wire System Coronary Artery Total Occlusion Study
Principal Investigator CREDO Trial
Principal Investigator Novoste CUP Trial
Principal Investigator CVD Accucath Infusion Catheter
Principal Investigator Duett Closure Devise
Principal Investigator EndoSonics Cath scanner Oracle - PTCA Catheter

Principal Investigator EPI FilterWire EX™ System During Transluminal Intervention of Saphenous Vein Grafts
Principal Investigator Extra Stent
Principal Investigator GREAT - Guided Radio Frequency Energy Ablation of Total Occlusions Using the Safe Cross™ Radio Frequency Total Occlusion Crossing System
Principal Investigator GRIP - Guided Radio Frequency in Peripheral Total Occlusions using the Safe-Cross™ Radio Frequency (RF) Total Occlusion (TO) Crossing System
Principal Investigator HIPS Trial
Principal Investigator Human Percutaneous Laser Angioplasty of the Coronary Arteries
Principal Investigator Johnson & Johnson Intracoronary Stent Program Supplement #27 "New" Delivery System
Principal Investigator Kensey Nash Hemostatic Puncture Closure Device
Principal Investigator Mansfield-Boston Scientific Strecker Coronary Stent
Principal Investigator Medtronic AVE S7 with Discrete Technology Coronary Stent System
Principal Investigator Medtronic AVE S7 Coronary Stent Registry
Principal Investigator MOBILE Trial - More Patency with Beta for In-Stent Restenosis in the Lower Extremities Trial IDE #G010295; Protocol D00789 Rev B dated 12/01
Principal Investigator NIR Stent Trial
Principal Investigator Neurex/Elan Pharmaceuticals Trial
Principal Investigator PAMI Stent Trial
Principal Investigator Paragon Stent
Principal Investigator Paris Radiation Trial
Principal Investigator PaS Trial
Principal Investigator Percutaneous Coronary Angioscopy in Unstable Angina
Principal Investigator Percutaneous Recanalization of Stenotic Human Coronary Arteries with Balloon Expandable Intracoronary Stents
Principal Investigator Percutaneous Recanalization of Stenotic Human Saphenous Vein Bypass Graft with Balloon Expandable Intraluminal Stents
Principal Investigator Percutaneous Thermal Balloon Angioplasty
Principal Investigator PMR Trial
Principal Investigator Pravastatin or Atorvastatin Evaluation and Infection Therapy (Prove It)
Principal Investigator Presto Trial
Principal Investigator RAVES Trial
Principal Investigator RESCUE Trial
Principal Investigator SAFER - Saphenous Vein Graft Angioplasty Free of Emboli Randomized Study Using the PercuSurge Guard Wire™ System
Principal Investigator SAVED Trial
Principal Investigator Schering-Plough Phase III Study of SCH 58235 in addition to Pravastatin compared to placebo in subjects with primary hypercholesterolemia
Principal Investigator Long-Term, Open-Label, Safety and Tolerability Study of SCH 58235 in Addition to Pravastatin in Patients with Primary Hypercholesterolemia
Principal Investigator Schneider WINS Trial
Principal Investigator SCORES Trial
Principal Investigator Sepracor Study of Norastemizole in Cardiac Compromised Subjects
Principal Investigator SMART Trial (National PI)
Principal Investigator SMART: Post-Approval Study
Principal Investigator SNAPIST - A Phase 2, Safety Study of Systemic Nanoparticle Paclitaxel (ABI-007) For In-Stent Restenosis; IND #63,082
Principal Investigator SOAR - Renal Stent
Principal Investigator Efficacy and Safety Study of the Oral Direct Thrombin Inhibitor H 376/95 Compared with Dose-Adjusted Warfarin (Coumadin) in the Prevention of Stroke and Systemic Embolic Events in Patients with Atrial Fibrillation (SPORTIF V)
Principal Investigator STARS Trial
Principal Investigator START Trial (National PI)
Principal Investigator STRATUS Trial
Principal Investigator STRESS III Trial

Principal Investigator	SUMO Trial
Principal Investigator	(SWING) Sound Wave Inhibition of Neointimal Growth
Principal Investigator	Talent Endoluminal Graft (High Risk & Low Risk)
Principal Investigator	Talent Endoluminal Spring Stent-Graft System
Principal Investigator	Tenax-XR Coronary Stent System
Principal Investigator	TITAN Trial
Principal Investigator	Trimedyne Excimer Laser Assisted Percutaneous Coronary Angioplasty
Sub-Investigator	Trimdyne Percutaneous Eclipse Holmium Laser Coronary Angioplasty
Principal Investigator	VeGAS 2 Trial
Principal Investigator	Velocity Trial Principal Investigator - Venus Stent
Co-Investigator	WALLSTENT Study
Principal Investigator	WIKTOR Coronary Stent

PHARMACOLOGY RESEARCH:

Principal Investigator	Abbott rUK Trial
Principal Investigator	Ajinimoto Pharmaceuticals Double-Blind Placebo-Controlled Study of AT-1015 in Patients with Intermittent Claudication due to peripheral arterial disease
Sub-Investigator	Amgen, Inc. Anakinra Trial for Rheumatoid Arthritis
Principal Investigator	Astra Zeneca Pharmaceutical Trial to Evaluate the Safety and Efficacy of XXXX and Atorvastatin
Principal Investigator	Astra Zeneca Trial Open Label Dose Comparison Study to Evaluate the Safety and Efficacy of Rosuvastatin versus Atorvastatin, Pravastatin, and Simvastatin in Subjects with Hypercholesterolemia
Principal Investigator	Parke-Davis and Pfizer Randomized Open-Label Study Comparing the Efficacy of Once Daily Atorvastatin to Simvastatin in Hypercholesterolemic Patients
Principal Investigator	Pilot Study to Evaluate Intracoronary Administration of Activase for the Treatment of Intracoronary Thrombus
Principal Investigator	Artistic Trial
Principal Investigator	AstraZeneca Trial of Niaspan versus New Generation Statin for the Treatment of Type IIB and Type IV Hyperlipidemia
Principal Investigator	AstraZeneca Multicenter Trial for drug (XXX) and Atorvastatin for the Treatment of Hypercholesterolemia
Principal Investigator	BRAVO Trial
Principal Investigator	BioVail Angina & Hypertension Trial
Principal Investigator	CAPRICORN Trial
Principal Investigator	Challenge Trial
Sub-Investigator	Comparison of Lopentol and Omnipaque in Adult Angiocardiography
Sub-Investigator	Comparison of Intravenous Adenosine to Intravenous Placebo in Termination of Spontaneous or Induced Paroxysmal Supraventricular Tachycardia
Principal Investigator	Centocor Chimeric 7E3 Fab
Principal Investigator	COR Therapeutics Randomized Placebo-Controlled Dose Ranging Study of drug (XXXX) in Patients with Atherosclerotic Cardiovascular, Peripheral Vascular, and/or Cerebrovascular Disease
Sub-Investigator	Dose Response Study of Bucindolol in Patients with Congestive Heart Failure
Principal Investigator	Effects of Recombinant Human Superoxide Dismutase in Patients with Acute Myocardial Infarction Subject to Coronary Artery Reperfusion
Sub-Investigator	Eli Lilly - Agitation/Alzheimer's Trial
Principal Investigator	EPILOG Trial
Principal Investigator	ERASER Trial
Principal Investigator	GUSTO Trial
Principal Investigator	A multi-center, randomized, double blind, placebo-and-active controlled Parallel Group Dose-ranging Study of the HMG CoA Reductase Inhibitor, BMS-423526, in the treatment of Hyperlipidemia

Principal Investigator Study Lovastatin XL with MEVACOR in patients with hypercholesterolemia
Sub-Investigator Lovastatin Multi-Center Trial
Principal Investigator Extended Trial of Lovastatin XL for the treatment of hypercholesterolemia
Principal Investigator Multicenter Double-Blind Placebo controlled trial of drug (XXXX) in patients with Type 2 Diabetes and Congestive Heart Failure
Principal Investigator Effect of LDL-Cholesterol Lowering Beyond Currently Recommended Minimum Targets on coronary heart disease (CHD) Recurrence in patients with Pre-Existing CHD
Principal Investigator A Double-Blind, Multi-Center, Randomized, Placebo-Controlled, Parallel Group Dosing Study Evaluating the Effects of Nebivolol on Blood Pressure in Patients with Mild to moderate Hypertension, NEB 302
Principal Investigator Parallel Group Extension Study to Determine the Safety and Efficacy of Long-Term Nebivolol Exposure in Patients with Mild to Moderate Hypertension NEB 306,
Sub-Investigator NeoTherapeutics Alzheimer's Disease 2000
Sub-Investigator NeoTherapeutics Alzheimer's Disease 2001
Principal Investigator OCTAVE Trial
Sub-Investigator OCTAVE Trial
Principal Investigator Pfizer Phase II Multicenter, double-blind placebo controlled randomized parallel group dose ranging study of the safety of CP529,414 soft-gel capsules
Principal Investigator PLAC Trial
Principal Investigator Protocol 073 Trial
Principal Investigator Knoll Pharmaceutical Double-Blind Randomized Clinical Trial of Slow Release Propafenone (Rythmol-SR®) in the Prevention of Symptomatic Recurrences of Atrial Fibrillation
Principal Investigator PREVAIL - A Phase 2 Multicenter, Double-Blind Placebo-Controlled, Dose-Ranging Study to Evaluate the Safety and Efficacy of BO-653 in Prevention of Post-Angioplasty Restenosis in Stented Lesions
Principal Investigator PROVE-IT TIMI 22 - Pravastatin or Atorvastatin Evaluation and Infection Therapy
Principal Investigator PURSUIT Trial
Principal Investigator QUIET Trial
Principal Investigator RAFT Trial
Principal Investigator REPLACE Randomized Evaluation in PCI Linking Angiomax to reduce Clinical Events
Sub-Investigator Safety and Efficacy Study of Burroughs - Wellcome Tissue Plasminogen Activator in Patients with Acute Myocardial Infarction
Principal Investigator A 6-week, open-label, dose-comparison study to evaluate the safety and Efficacy of Rosuvastatin versus Atorvastatin, Cerivastatin, pravastatin, and Simvastatin in subjects with hypercholesterolemia
Principal Investigator A 48-week, open-label, non-comparative, Multicentre, Phase IIIb study to evaluate the efficacy and safety of the Lipid-Regulating agent Rosuvastatin in the treatment of subjects with Fredrickson Type IIa and Type IIb Dyslipidemia, including Heterozygous Familial Hypercholesterolemia
Principal Investigator SAGE Trial
Sub Investigator Long Term Open Label Safety and Tolerability Study of SCH58235 inn addition to Pravastatin in Patient With Primary Hypercholesterolemia
Principal Investigator Phase III Double-Blind Efficacy and Safety Study SCH58235 (10 mg) in Addition to Pravastatin Compared to Placebo in Subjects with Primary Hypercholesterolemia
Principal Investigator Phase III Open Label Efficacy and Safety Study SCH58235 (10 mg) in Addition to Pravastatin Compared to Placebo in Subjects with Primary Hypercholesterolemia
Principal Investigator Sepracor Protocol Study of Norastemizole in Cardiac Compromised Subjects
Principal Investigator SPORTIF V - Atrial Fibrillation Trial
Principal Investigator SWORD Trial
Principal Investigator Titration-to-Response Trial Comparing Micardis and COZAAR® in Patients with Mild-to-moderate Hypertension

Principal Investigator TNT Trial
Principal Investigator TREND Trial
Sub-Investigator VALDECOXIB Trial
Principal Investigator An Open-Label, Multinational, Multicentre, Extension Trial to Assess the Long-Term Safety and Efficacy of ZD4522 in Subjects in the ZD4522 Clinical Trial Program

BASIC RESEARCH:

1990 - 1993 Systematic assessment of Medtronic balloons and guiding catheters in porcine and canine models. Sponsored by Medtronic, Inc.

1990 - 1993 Determination of radiopacity and torquability of Medtronic vascular catheters in porcine models. Sponsored by Medtronic, Inc.

1992 - 1996 Evaluation of Strecker stent in porcine and canine models. Sponsored by Boston Scientific

Evaluation of Wiktor stent and stent in porcine and canine models. Sponsored by Medtronic, Inc.

Evaluation of NIR stent in porcine models. Sponsored by Cordis Corp.

1990 - 1994 Evaluation of Japan Crescent radiofrequency balloon in porcine model with emphasis on histopathology of heat-produced lesions. Abstract submitted at 1993 AHA Conference.

1993 Evaluation of radiofrequency wire for total coronary occlusions in porcine models: Determining energy limitations. Equipment subsequently licensed to Radius Medical.

1994 - 1997 Training courses for professionals (physicians, engineers, technicians) in techniques and strategies for placement of coronary stents. Five courses sponsored by Johnson & Johnson, Medtronic, Inc. and Cook, Inc.

1997 Efficacy of the Endotex Abdominal Aortic Aneurysm exclusion device in a porcine model gauging ability to exclude renal arteries, ease of placement and radiopacity. Sponsored by Endotex

1998 Use of percutaneous myocardial revascularization in a porcine model. Sponsored by Cardiogenesis Corporation at Stanford University.

1998 - 1999 Utility of radiofrequency (RF) percutaneous myocardial revascularization in acute and chronic porcine model: Histopathology and angiogenesis related to use of RF alone and in combination with growth factor (VEGF). Results presented at Angiogenesis 1999, Washington, DC.

1999 Development and testing of embolic probe device in porcine model (patent pending). Performed at PRMC and separately at Columbia Presbyterian in New York.

1999 Evaluation of the Medtronic carotid and SVG stent in porcine carotid and saphenous vein graft lesions assessing ease of use and 30-day outcome. Sponsored by Medtronic, Inc.

1999 Development and testing of Protector vascular embolic protection device in

porcine model at Mayo Clinic (device patent pending).

1999 Evaluation of ability of intramuscular growth factor to stimulate angiogenesis in rabbit hindlimb model at 30 and 60 days post-procedure. Sponsored by Sulzer Medical.

1999 Use of *Vesseal* device to close porcine peripheral artery tears (patent #6,159,197) Sponsored by Phoenix Heart Center.

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Heuser RR, Mehta SS: Holmium laser angioplasty after failed coronary balloon dilation: Use of a new solid-state, infrared laser system. Catheterization and Cardiovascular Diagnosis 1991 Jul;23(3):187-189.

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Heuser RR: The use of the Holmium: YAG laser in coronary disease: The utility of a unique lensed fiber catheter. The Journal of Interventional Cardiology 1992 Dec;5(4):293-300.

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AWARDS & HONORS:

Columbia/HCA Cardiovascular Management Network - 1998 Cardiologist of the Year

PATENTS:

1. Method and Apparatus for Treating Body Tissues and Bodily Fluids; Patent granted December 12, 2000 Number: 6,159,197
2. Hot Tip Catheter; Patent granted February 20, 2001 Number: 6,190,379
3. Embolism Prevention Device; Patent granted April 2, 2002 Number: 6,364,900
4. Catheter apparatus and Method for Arterializing a Vein; Patent granted October 15, 2002 Number 6,464,665
5. Methods and apparatus for treating body tissues and bodily fluid vessels; Patent granted October 15, 2002 Number: 6,464,681
6. Catheter for Thermal Evaluation of Arteriosclerotic Plaque; Patent granted March 25, 2003 Number: 6,536,949
7. Small Diameter Snare; Patent granted April 29, 2003 Number: 6,554,842

EXHIBIT
B

DISCLOSURES

APPLICATION
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EXHIBIT B

DISCLOSURES
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Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell

nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles

and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

EXHIBIT C

DEFINITIONS

EXHIBIT C

DEFINITIONS

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

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> **Growth factor:** a substance (as a vitamin B_{12} or an interleukin) that promotes growth and especially cellular growth

EXHIBIT D

CLAIMS

EXHIBIT D

CLAIMS

Claim X: **A method for growing a new portion of a pre-existing heart comprising the steps of: placing a growth factor in a body of a human patient and growing new muscle in said heart.**

EXHIBIT E

PUBLICATIONS

EXHIBIT E

PUBLICATION INFORMATION SUMMARY

TITLE	AUTHOR	CITATION	DATE	AUTHOR COUNTRY	ROUTE OF ADMINISTRATION	GROWTH FACTOR ADMINISTERED	RESULT
Left Ventricular Electromechanical Mapping to Assess Efficacy of phVEGF165 Gene Transfer for Therapeutic Angiogenesis in Chronic Myocardial Ischemia	Vale	Circulation. 2000; 102:965-974	08/29/00	U.S.	Small incision (minithoracotomy) with syringe injection	VEGF (Gene form)	Repair of damaged portion of heart – Also pertains to new muscle growth
Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans	Strauer	Circulation. 2002; 106:1913-1918	10/08/02	Germany	Balloon catheter with injection	Bone Marrow Cells	Repair of dead portion of heart – also pertains to new muscle growth

TITLE	AUTHOR	CITATION	DATE	AUTHOR COUNTRY	ROUTE OF ADMINISTRATION	GROWTH FACTOR ADMINISTERED	RESULT
Viability and differentiation of autologous skeletal myoblast grafts in ischemic cardiomyopathy	Hagege	Lancet 2003 Feb 8; 361 (9356):491-492	2003	France	Injection	Skeletal Muscle Cells	Repair of dead portion of heart; Histological Proof (muscle)
Autologous Cell Transplant Helpful in Ischemic Heart or Legs	Barclay	Medscape Medical News 2000 – Abstract from American Heart Association's 75th Scientific Sessions on 11/18/02, Chicago	11/18/02	U.S.	Surgery with syringe injection	Bone Marrow Cells	Repair of damaged portion of heart – also pertains to new muscle growth
Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation	Pagani	J Am Coll Cardiol 2003 Mar 5; 41(5): 879-888	2003	U.S.	Surgery with syringe injection	Skeletal Muscle Cells	Repair of dead portion of heart; Histological Proof (muscle and blood vessels)

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

Bodo E. Strauer, MD; Michael Brehm, MD; Tobias Zeus, MD; Matthias Köstering, MD; Anna Hernandez, PhD; Rüdiger V. Sorg, PhD; Gesine Kögler, PhD; Peter Wernet, MD

Background—Experimental data suggest that bone marrow–derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30±13 to 12±7%, $P=0.005$) and was also significantly smaller compared with the standard therapy group ($P=0.04$). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, $P=0.028$). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (*Circulation.* 2002;106:1913-1918.)

Key Words: myocardial infarction ■ cell transplantation, intracoronary ■ angiogenesis ■ bone marrow ■ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.[1,2] Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.[3]

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.[4-7] Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow–derived cells. Bone marrow contains multipotent adult stem cells that show a high capacity for differentiation.[8-10] Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.[11-14] However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.[15] Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.[16] However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

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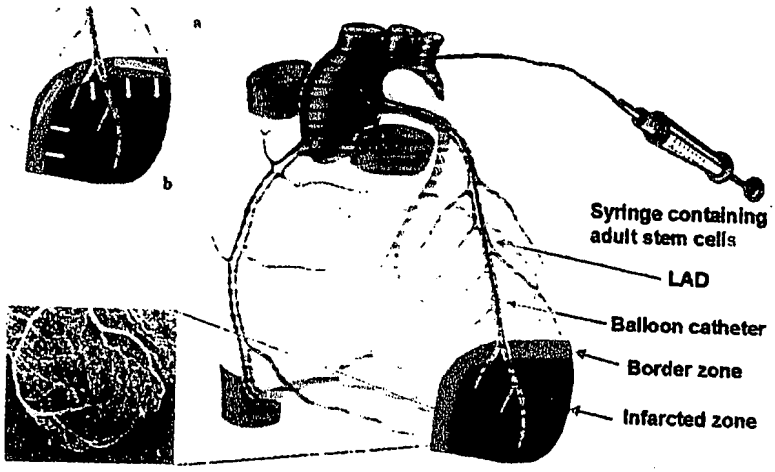

Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. a, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. b, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. c, A supply of blood flow exists within the infarcted zone.[35] The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.[17] To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery (n=4), left circumflex coronary artery (n=3), or right coronary artery (n=13). Mean duration of infarct pain was 12±10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty (n=20) and subsequent stent implantation (n=19). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β-blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (±2) days after acute coronary angiography, bone marrow (~40 mL) was aspirated under local anesthesia from ilium of cell therapy patients (n=10). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H_2O. For overnight cultivation, 1×10^6 BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was 93±3%. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10^7; this consisted of 0.65±0.4% AC133-positive cells and 2.1±0.28% CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality ex vivo control, 1×10^5 cells grown in H5100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.[18]

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.[19] All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radionuclide ventriculography, catheterization of the right heart, and

TABLE 1. Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	*P*
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2	...	...
Mononuclear bone marrow cells, n ($\times 10^7$)	2.8±2.2	...	...

Values are mean±SD or number of patients.
NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection ^{201}thallium scintigraphy. The contractility index P_{sys}/ESV was calculated by dividing LV systolic pressure (P_{sys}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypokinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, $P=0.005$). It was also significantly smaller compared with the standard therapy group after 3 months ($P=0.04$). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, $P=0.028$) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, P=NS). No significant difference was observed between the two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm², $P=0.016$, assessed by ^{201}thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

(1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m², $P=0.010$) and ejection fraction (from 51±14 to 53±13%, P=NS).
(2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, P=NS) and end-systolic volume (from 82±26 to 67±21 mL, $P=0.011$). Radionuclide ventriculography was used to acquire the data.
(3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, P=NS, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, $P=0.005$).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	*P*
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dyskinetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dyskinetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow–derived cells in experimental infarction.[11–14,18,20–23] Moreover, transendothelial migration from the coronary capillaries and incorporation of cells into heart muscle has been observed experimentally.[3,12,24–26] Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.[15,16] Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.[27] Adult, mononuclear BMCs contain such stem and progenitor cells (≤1%), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

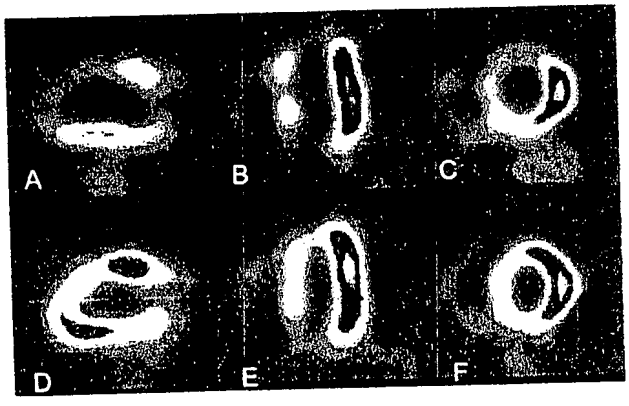

Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by ^{201}thallium scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	*P*
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume index, mL/m^2	49±7	56±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P_{sys}/ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
^{201}Thallium scintigraphy, cm^2	174±99	128±71	0.016

NS indicates not significant.

and smooth muscle cells[8-13]; (3) BMCs give rise to mesodermal progenitor cells that differentiate to endothelial cells[28]; and (4) endothelial progenitors can transdifferentiate into beating cardiomyocytes.[29] Thus, several different fractions of mononuclear BMCs may contribute to the regeneration of necrotic myocardium and vessels. In order to utilize this large and perhaps heterogeneous regenerative potential, we decided to use all mononuclear cells from the bone marrow aspirate as a whole, rather than a subpopulation. No further expansion was performed because experimental data have revealed a dramatic decline in the homing capacity of in vitro amplified hematopoietic stem or progenitor cells.[30]

The second question was how to deliver the cells most efficiently. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection: assuming a normal coronary blood flow of 80 mL/min per 100 g of LV weight, a quantity of 160 mL per left ventricle (assuming a regular LV mass of ≈200 g) will flow per minute.[31,32] This corresponds to only about 3% of cardiac output (assuming a cardiac output of 5000 mL/min).[31] Therefore, intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Thus, supplying the entire complement of cells by intracoronary administration obviously seems to be advantageous for the tissue repair of infarcted heart muscle and may also be superior to intraventricular injection,[33] because all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage. Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched with the maximum available amount of cells at all times.

As stem cells differentiate into more mature types of progenitor cells, it is thought that a special microenvironment in so-called niches regulates cell activity by providing specific combinations of cytokines and by establishing direct cellular contact. For successful long-term engraftment, at least some stem cells have to reach their niches, a process referred to as homing. Mouse experiments have shown that significant numbers of BMCs appear in liver, spleen, and bone marrow after intravenous injection.[34] To offer the BMCs the best chance of finding their niche within the myocardium, a selective intracoronary delivery route was chosen. Presumably, therefore, fewer cells were lost by extraction toward organs of secondary interest by this first pass–like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area, and the balloon was kept inflated for 2 to 3 minutes; the cells were not washed away immediately under these conditions.

The time point for delivery was chosen as 7 to 8 days after infarction onset for the following reasons:

(1) In dogs, infarcted territory becomes rich in capillaries and contains enlarged, pericyte-poor "mother vessels" and endothelial bridges 7 days after myocardial ischemia and reperfusion. Twenty-eight days later, a significant muscular vessel wall has already formed.[35] Thus, with such timing, cells may be able to reach the worst damaged parts and at the same time salvage tissue. Transendothelial cell migration may also be enhanced because an adequate muscular coat is not yet formed.

(2) Until now, only one animal study has attempted to determine the optimum time for cardiomyocyte transplantation to maximize myocardial function after LV injury. Adult rat hearts were cryoinjured and fetal rat cardiomyocytes were transplanted immediately, 2 weeks later, and 4 weeks later. The authors discussed the inflammatory process, which is strongest in the first days after infarction, as being responsible for the negative results after immediate cell transplantation, and they assumed that the best results seen after 2 weeks may have been due to transplantation before scar expansion.[36] Until now, however, no systematic experiments have been performed with BMCs to correlate the results of transplantation with the length of such a time delay.

(3) Another important variable is the inflammatory response in MI, which seems to be a superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. In the first 48 hours, debridement and formation of a fibrin-based provisional matrix predominates before a healing phase ensues.[37-40] Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the decline of adhesion molecules (intercellular adhesion molecules, vascular cell adhesion molecules) does not take place before days 3 to 4 after MI. We assumed that transplantation of mononuclear BMCs within the "hot" phase of post-MI inflammation might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels.

Taking all of this into account, we can conclude that cell transplantation within the first 5 days after acute infarction is not possible for logistical reasons and is not advisable because of the inflammatory process. On the other hand, transplantation 2 weeks after infarction scar formation seems to reduce the benefit of cell transplantation. Although the ideal time point for transplantation remains to be defined, it is most likely between days 7 and 14 after the onset of MI, as in the present study.

This trial was designed as a phase I safety and feasibility trial, meaning that no control group is necessarily required. However, to validate the results, we correlated them with those obtained from 10 patients who refused to get additional cell therapy and thus received standard therapy alone. We are aware of the fact that such a comparison does not reach the power of a randomly allocated, blinded control group. However, the significant improvement with regard to infarct region, hemodynamics (stroke volume index), cardiac geometry (LV end-systolic volume), and contractility (P_{syst}/ESV and infarction wall movement velocity) did confirm a positive effect of the additional cell therapy because the changes observed in the standard therapy group failed to reach significance.

Another important factor for interpreting the results is time interval between onset of symptoms and revascularization of the infarct-related artery by angioplasty; this represents a crucial determinant of LV recovery. For patients with acute MI, it has

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.[41] None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12±10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.[41] In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angioneogenesis and cardiomyoneogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

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1: Lancet 2003 Feb 8;361(9356):491-2

ELSEVIER SCIENCE
FULL-TEXT ARTICLE

Viability and differentiation of autologous skeletal myoblast grafts in ischaemic cardiomyopathy.

Hagege AA, Carrion C, Menasche P, Vilquin JT, Duboc D, Marolleau JP, Desnos M, Bruneval P.

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Autologous skeletal myoblast transplantation might improve postinfarction ventricular function, but graft viability and differentiation (ie, proof of concept) has not been shown. A 72-year-old man had autologous cultured myoblasts from his vastus lateralis injected to an area of transmural inferior myocardial infarction in non-reperfused scar tissue. He showed improvement in symptoms and left-ventricular ejection fraction. When he died 17.5 months after the procedure, the grafted post-infarction scar showed well developed skeletal myotubes with a preserved contractile apparatus. 65% of myotubes expressed the slow myosin isoform and 33% coexpressed the slow and fast isoforms (vs 44% and 0.6%, respectively, in skeletal muscle). Myoblast grafts can survive and show a switch to slow-twitch fibres, which might allow sustained improvement in cardiac function.

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myocardial tissue. These results establish the feasibility of myoblast transplants for myocardial repair in humans.

Publication Types:
- Clinical Trial
- Clinical Trial, Phase I

PMID: 12628737 [PubMed - indexed for MEDLINE]

Medscape Medical News

Autologous Cell Transplant Helpful in Ischemic **Heart** or Legs

Laurie Barclay, MD

Nov. 18, 2002 — Autologous cell transplantion may benefit ischemic hearts and legs, according to three presentations on Nov. 18 at the American **Heart** Association's 75th Scientific Sessions held in Chicago, Illinois. Two studies focused on injecting autologous bone marrow cells or autologous skeletal myoblasts into the scarred area of an infarcted **heart**. In another study, injecting autologous bone marrow into ischemic limbs led to new vessel growth, reducing the need for amputation.

"Bone marrow not only can differentiate into **heart** cells, but also smooth muscle cells, connective **tissue** cells and other types of cells to reconstitute the entire structure of a **tissue**," presenter Manuel Galinanes, MD, from the University of Leicester in the U.K., says in a news release. "The benefit [of transplanting bone marrow into scar **tissue** of the **heart**] could be seen only six weeks **after** injection."

In 14 patients with low ejection fraction post-myocardial infarction (MI), autologous bone marrow from the sternum was injected into scarred myocardium during nonemergency coronary artery bypass surgery. **Heart** wall motion measured with echocardiography improved within weeks of treatment, and improvements persisted for at least 10 months **after** treatment.

The regional wall motion score decreased significantly, reflecting less movement abnormality, from a mean score of 2.41 at baseline to 2.16 six weeks **after** treatment and 2.09 ten months **after** treatment. The global wall motion score also decreased significantly from 1.96 before surgery to 1.64 at six weeks, and stabilized at 1.65 **after** 10 months.

Although it is still unproven that bone marrow creates a new cellular infrastructure in **heart** scar **tissue**, "that is the only possible explanation," Galinanes says. "The ability to confirm the presence of scar **tissue** with dobutamine stress echo before surgery, and then confirm it again during surgery, told us that the affected area was dysfunctional and the abnormality was irreversible. We wanted to make sure that we were injecting the marrow into **dead tissue** to help ensure that the injection would not pose any serious risk to the patient."

If additional studies confirm safety and efficacy, Galinanes says that this treatment would be a welcome addition to the post-MI arsenal, which also includes gene therapy, growth factor therapy, and laser treatments.

In a multicenter trial supervised by the U.S. Food and Drug Administration, investigators safely transplanted 16 patients with autologous skeletal myoblasts injected into hearts severely damaged by MI or **heart** failure. Baseline left-ventricular ejection fraction was less than 30%. Eleven patients were undergoing coronary artery bypass surgery and five were having implantation of a left ventricular assist device. Myoblasts extracted from thigh muscle were grown in large quantities in vitro using a controlled cell expansion manufacturing process, and were injected in doses ranging from 10 million to 300 million cells.

"We have been able to **regenerate dead heart** muscle, or scar **tissue**, in the area of **heart** attack without increasing risk of death. Our findings will allow us to move forward with testing if the procedure can improve the contractility of the **heart**," says lead author Nabil Dib, MD, from the Arizona **Heart** Institute in Phoenix. "We found that the transplanted myoblasts survived and thrived in patients. Areas damaged by **heart** attack and cardiovascular disease showed evidence of repair and viability."

Twelve weeks **after** transplant, mean ejection fraction rates improved from 22.7% to 35.8%, or a 58% increase. Echocardiogram, magnetic resonance imaging, and positron emission tomography showed evidence of regeneration in the area of the graft. There were no significant adverse events related to the cell transplant procedure at nine-month follow-up.

The third study showed that bone marrow cells implanted into ischemic legs in patients with peripheral arterial disease (PAD) formed new blood vessels, increased blood flow, and prevented amputation.

"This is the first multicenter and double-blind clinical study to prove the clinical efficacy of growing new blood vessels (angiogenesis) using bone marrow cell transplantation," says lead author Hiroya Masaki, MD, PhD, from Kansai Medical University in Osaka, Japan.

In this randomized trial, 45 patients with PAD received injections of autologous bone marrow mononuclear cells into the calf muscles. Compared with controls who received saline injections, patients who received bone marrow mononuclear cell transplants had a "striking" increase in new capillary formation and in newly visible collateral vessels.

Of 45 treated patients, 31 had an increase in ankle-brachial pressure index in the treated limbs, and 39 had decreased rest pain with improved treadmill endurance. Ischemic ulcers or gangrene healed in 21 of 28 treated limbs.

CD34-cells, which can develop into endothelial progenitor cells, expressed angiogenic growth factors including basic fibroblast growth factor, vascular endothelial growth factor, and angiopoietin-1. Although more research is needed to determine long-term efficacy and safety, "this new angiogenesis therapy using bone marrow cell transplantation may help many patients suffering with ischemic limbs," Masaki says.

AHA 75th Scientific Sessions: Abstracts 111623, 101758, 109801. Presented Nov. 18, 2002.

Reviewed by Gary D. Vogin, MD

1: J Am Coll Cardiol 2003 Mar 5;41(5):879-88

Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation.

Pagani FD, DerSimonian H, Zawadzka A, Wetzel K, Edge AS, Jacoby DB, Dinsmore JH, Wright S, Aretz TH, Eisen HJ, Aaronson KD.

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OBJECTIVES: We report histological analysis of hearts from patients with end-stage heart disease who were transplanted with autologous skeletal myoblasts concurrent with left ventricular assist device (LVAD) implantation. BACKGROUND: Autologous skeletal myoblast transplantation is under investigation as a means to repair infarcted myocardium. To date, there is only indirect evidence to suggest survival of skeletal muscle in humans. METHODS: Five patients (all male; median age 60 years) with ischemic cardiomyopathy, refractory heart failure, and listed for heart transplantation underwent muscle biopsy from the quadriceps muscle. The muscle specimen was shipped to a cell isolation facility where myoblasts were isolated and grown. Patients received a transplant of 300 million cells concomitant with LVAD implantation. Four patients underwent LVAD explant after 68, 91, 141, and 191 days of LVAD support (three transplant, one LVAD death), respectively. One patient remains alive on LVAD support awaiting heart transplantation. RESULTS: Skeletal muscle cell survival and differentiation into mature myofibers were directly demonstrated in scarred myocardium from three of the four explanted hearts using an antibody against skeletal muscle-specific myosin heavy chain. An increase in small vessel formation was observed in one of three patients at the site of surviving myotubes, but not in adjacent tissue devoid of engrafted cells. CONCLUSIONS: These findings represent demonstration of autologous myoblast cell survival in human heart. The implanted skeletal myoblasts formed viable grafts in heavily scarred human

2.89

CERTIFICATE OF MAILING

I hereby certify that the attached AMENDMENT (with Exhibits I-IX and two IDS documents); Supplemental Declaration of Richard Heuser; and Supplemental Declaration of Andrew E. Lorincz were delivered to the Commissioner for Patents by the undersigned from Arrow Intellectual Property Services, 2001, Jefferson Davis Highway, Suite 602, Arlington, Virginia 22202, by hand carrying said AMENDMENT to ~~Art Unit 1646~~ MAIL Room, Attention: Examiner Elizabeth C. Kemmerer this 17th day of February, 2004.

Dated: 2/17/04

Ann Rutledge

Printed Name: Ann Rutledge

ARROW INTELLECTUAL PROPERTY SERVICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia	)	
	)	
SERIAL NO.: 09/836,750	)	EXAMINER: E.C. Kemmerer, Ph.D.
	)	
FILED: April 17, 2001	)	
	)	GROUP ART UNIT: 1646
FOR: METHOD FOR GROWING MUSCLE IN A HUMAN HEART	)	

SUPPLEMENTAL DECLARATION OF RICHARD HEUSER, M.D.

I, Richard Heuser, declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.

2. This Supplemental Declaration is submitted in addition to my previously submitted Declaration in this application, dated June 5, 2003, and makes no changes to such previous Declaration.

3. My Curriculum Vitae is attached as Exhibit A to my previous Declaration.

4. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. Such disclosures are the same as read and understood by me in my previous Declaration. A copy of such disclosures is attached hereto as Supplemental Exhibit A.

5. I note that the disclosures referenced in above Paragraph 4 relate to using a growth factor for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new muscle in the heart.

6. I have read and understood the claims set forth in Supplemental Exhibit B and have been informed that such claims will be presented to the Patent and Trademark Office in the near future.

7. Based upon above Paragraphs 4-6 and Paragraph 7 of my previous Declaration, it is my opinion that introducing a growth factor into a human patient will predictably cause new muscle growth in the heart of the patient.

8. Based upon above Paragraphs 4-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Supplemental Exhibit B without need for resorting to undue experimentation. I have been informed that the Examiner has questioned the fact that dosages are not recited in the specification of the above-identified application in connection with the administration of cell growth factors to a human patient with use of intravenous or intraluminal techniques. Such techniques are the subject of claims 248-249 in above-mentioned Supplemental Exhibit B. In my opinion, dosages of cellular growth factors to achieve the above-mentioned heart muscle growth are a matter of routine medical practice, requiring only a reasonable degree of experimentation, depending upon such factors as extent of prior heart condition, size of patient, age of patient, health of patient, etc. Consequently, it is my opinion that the disclosure mentioned in Supplemental Exhibit A would enable a person skilled in the medical arts to practice the invention of claims 248-249 and predictably anticipate the results defined therein without need for resorting to undue experimentation.

9. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2/4/04

Richard Heuser

SUPPLEMENTAL EXHIBIT A

DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell

nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles

and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

SUPPLEMENTAL EXHIBIT B

CLAIMS
APPLICATION SERIAL NO. 09/836,750

236. A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new muscle and growing a new artery in said heart.

238. The method of claim 236, further comprising repairing a dead portion of said heart.

239. The method of claim 236, further comprising repairing a damaged portion of said heart.

240. The method of claim 236, wherein said growth factor comprises genetic material selected from the group consisting of a portion of a gene, a gene, a gene product, and an extracellular matrix.

241. The method of claim 240, wherein said genetic material comprises a gene.

242. The method of claim 241, wherein said gene comprises VEGF.

243. The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.

244. The method of claim 243, wherein said growth factor comprises a cell

245. The method of claim 244, wherein said cell is multifactorial and non-specific.

246. The method of claim 245, wherein said cell comprises a stem cell.

247. The method of claim 236, wherein said growth factor is placed in said patient by injection.

248. The method of claim 247, wherein said injection is intravenous.

249. The method of claim 247, wherein said injection is intraluminal.

250. The method of claim 247, wherein said injection is intramuscular.

251. The method of claim 236, wherein said growth factor is placed in said patient by a carrier.

252. The method of claim 251, wherein said carrier comprises an angioplasty balloon.

253. The method of claim 236, wherein said growth factor comprises a gene and a cell.

Docket No. 1000-10-C01

Serial No. 09/836,750

Filed 04/17/01

Due Date: - - -

The Patent Office acknowledges, and has stamped hereon, the date of receipt of the items check below:

☒ Transmittal Letter	
☐ Application – Trademark	
☐ Application – Patent Specification	Total Pgs____
☐ Total Claims____ Ind. Claims____	Total Pgs____
☐ Abstract	Total Pgs____
☐ Drawings: Formal ___ Informal ___	Total Pgs____
☐ Declaration/Oath/Power of Attorney	Total Pgs____
☐ Check No. _____	Fee: $_____
☐ Assignment and Cover Sheet	Fee: $_____
☐ Request for Non-Publication	
☐ Information Disclosure Statement	
☐ Form PTO-1449 w/Refs_____	Total No. ____
☐ Request for Extension of Time	Fee: $_____
☐ Amendment/Response	
☒ Affidavit/Declaration	2nd Supplemental of Drs. Heuser and Lorincz
☐ Brief/Reply Brief/Notice of Appeal	
☐ Fee-Base/Maintenance	Fee: $_____
☐ Check No. _____	
☒ w/Exhibits A - B	
☐ _____	

07/26/04

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia)
)
Serial No.: 09/836,750)
)
Filed: April 17, 2001)
)
For: METHOD FOR GROWING)
MUSCLE IN A HUMAN HEART)

Group Art Unit: 1646

Examiner: Elizabeth C. Kemmerer, Ph.D.

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail, in an envelope addressed to Mail Stop Non-Fee Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on July 26, 2004

Gerald K. White 7/26/04
Signature Date

LETTER

Mail Stop Non-Fee Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This Letter is submitted in an attempt to advance the prosecution of the instant patent application.

In related patent application Serial No. 09/794,456, that is currently being examined by the Examiner in charge of this application, Second Supplemental Declarations of Drs. Richard Heuser and Andrew E. Lorincz were prepared and will be filed in the near future. Accordingly, Applicant wishes to file Second Supplemental Declarations in this application as well.

Should the Examiner have any questions or require additional information or discussion to place the application in condition for allowance, a phone call to the undersigned attorney would be appreciated.

Respectfully submitted,

Date: July 26, 2004

Gerald K. White
Reg. No. 26,611
Attorney for Applicant

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)
)
SERIAL NO.: 09/836,750) EXAMINER: E.C. Kemmerer, Ph.D.
)
FILED: April 17, 2001)
) GROUP ART UNIT: 1646
FOR: METHOD FOR GROWING)
MUSCLE IN A HUMAN HEART)

SECOND SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.

I, Richard Heuser, declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.

2. This Second Supplemental Declaration is submitted in addition to my previous Declaration, dated June 5, 2003 and my Supplemental Declaration dated February 4, 2004. No changes are made to either of such previous Declarations.

3. My Curriculum Vitae (hereinafter "CV") is attached as Exhibit A to my Declaration of June 5, 2003.

4. It is my understanding that the Examiner in charge of the above-identified patent

application, in an Office Action dated June 1, 2004 for related patent application Serial No. 09/794,456, questioned my qualification, for the first time, to render my previous opinions mentioned in above Paragraph 2. It is my further understanding that the basis for such questioning was that the Examiner noted that I did not report experience with cellular therapy. I desire to provide the information contained in following paragraph 5 so that the Examiner can consider such information in this application, as well.

5. I am currently Director of Cardiovascular Research at St. Joseph's Hospital and Medicine Center, and I serve as Clinical Professor of Medicine at University of Arizona College of Medicine. Over the past six years, I have worked in gene therapy, as well as muscle regeneration for the treatment of cardiomyopathy.

In my CV, you will note reference to work that was done with Sulzer Medical involving a rabbit hind limb model to stimulate peripheral vascular disease. I injected a growth mixture that included FGF, etc. into the hind limb model.

In my U.S. Patent No. 6,190,379 entitled "Hot Tip Catheter," I developed a technique to deliver radiofrequency (PMR). In the full embodiment of the patent, I discuss delivery of protein and/or muscle cells in the myocardium using the inventive technique.

I have been involved as a member of the scientific advisory board with the world leader in cardiomyocyte regeneration, Bioheart, Miami Lakes, Florida. This company has been involved with laboratory and clinical trials using skeletal muscle cultured and modified. The sample is then delivered into the myocardium via a surgical or catheter approach.

6. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 17; and page 44, line 19 through page 46, line 16. Such disclosures are the same as I read and understood in my previous Declaration and

Supplemental Declaration. A copy of such disclosures is attached hereto as Second Supplemental Declaration Exhibit A.

7. I note that the disclosures referenced in above Paragraph 6 relate to using a growth factor for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new cardiac muscle in the heart.

8. I have read and understood the claims set forth in Second Supplemental Declaration Exhibit B and have been informed that such claims are currently presented in this application.

9. Based upon above Paragraphs 6-8, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be enabled to practice the method set forth in Second Supplemental Declaration Exhibit B and to predictably anticipate the results defined therein without need for resorting to undue experimentation.

10. I believe that one skilled in the medical arts, upon reading the disclosures in above , such as multifactorial and non-specific cells,Paragraph 6, would understand that cellular growth factors are included in such disclosures. Moreover, such skilled person would understand the disclosure on page 45 to be authored as an illustration of various modes of delivery of growth factors, whether they are genes or other genetic material; and that such skilled person would further understand that the disclosures on pages 45 and 46 describe genetic material to include appropriate cells and genes.

11. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 7/18/04

Richard Heuser, M.D., F.A.C.C., F.A.C.P.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT A

DISCLOSURES

SECOND SUPPLEMENTAL DECLARATION
EXHIBIT A

DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or

other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in

connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the

heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT B

CLAIMS

SECOND SUPPLEMENTAL DECLARATION
EXHIBIT B

CLAIMS
APPLICATION SERIAL NO. 09/836,750

236. A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new cardiac muscle and growing a new artery in said heart.

238. The method of claim 236, further comprising repairing a dead portion of said heart.

239. The method of claim 236, further comprising repairing a damaged portion of said heart.

240. The method of claim 236, wherein said growth factor comprises genetic material selected from the group consisting of a portion of a gene, a gene, a gene product, and an extracellular matrix.

241. The method of claim 240, wherein said genetic material comprises a gene.

242. The method of claim 241, wherein said gene comprises VEGF.

243. The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.

244. The method of claim 243, wherein said growth factor comprises a cell

245. The method of claim 244, wherein said cell is multifactorial and non-specific.

246. The method of claim 245, wherein said cell comprises a stem cell.

247. The method of claim 236, wherein said growth factor is placed in said patient by injection.

248. The method of claim 247, wherein said injection is intravenous.

249. The method of claim 247, wherein said injection is intraluminal.

250. The method of claim 247, wherein said injection is intramuscular.

251. The method of claim 236, wherein said growth factor is placed in said patient by a carrier.

252. The method of claim 251, wherein said carrier comprises an angioplasty balloon.

253. The method of claim 236, wherein said growth factor comprises a gene and a cell.

254. A method of growing a new portion of a pre-existing organ comprising placing a growth factor in a body of a patient to grow new muscle in said organ.

255. The method of claim 254, wherein said organ comprises a heart.

256. The method of claim 255, wherein said new muscle comprises cardiac muscle and said growth factor comprises a stem cell.